

# Hepatocyte growth factor inhibits hypoxia/reoxygenation-induced activation of xanthine oxidase in endothelial cells through the JAK2 signaling pathway

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**Abstract.** Vascular endothelial cells (ECs) appear to be one of the primary targets of hypoxia/reoxygenation (H/R) injury. In our previous study, we demonstrated that hepatocyte growth factor (HGF) exhibited a protective effect in cardiac microvascular endothelial cells (CMECs) subjected to H/R by inhibiting xanthine oxidase (XO) by reducing the cytosolic  $\text{Ca}^{2+}$  concentration increased in response to H/R. The precise mechanisms through which HGF inhibits XO activation remain to be determined. In the present study, we examined the signaling pathway through which HGF regulates  $\text{Ca}^{2+}$  concentrations and the activation of XO during H/R in primary cultured rat CMECs. CMECs were exposed to 4 h of hypoxia and 1 h of reoxygenation. The protein expression of XO and the activation of the phosphoinositide 3-kinase (PI3K), janus kinase 2 (JAK2) and p38 mitogen-activated protein kinase (p38 MAPK) signaling pathways were detected by western blot analysis. Cytosolic calcium ( $\text{Ca}^{2+}$ ) concentrations and reactive oxygen species (ROS) levels were measured by flow cytometry. The small interfering RNA (siRNA)-mediated knockdown of XO inhibited the increase in ROS production induced by H/R. LY294002 and AG490 inhibited the H/R-induced increase in the production and activation of XO. The PI3K and JAK2 signaling pathways were activated by H/R. The siRNA-mediated knockdown of PI3K and JAK2 also inhibited the increase in the production of XO protein.

HGF inhibited JAK2 activation whereas it had no effect on PI3K activation. The siRNA-mediated knockdown of JAK2 prevented the increase in cytosolic  $\text{Ca}^{2+}$  induced by H/R. Taken together, these findings suggest that H/R induces the production and activation of XO through the JAK2 and PI3K signaling pathways. Furthermore, HGF prevents XO activation following H/R primarily by inhibiting the JAK2 signaling pathway and in turn, inhibiting the increase in cytosolic  $\text{Ca}^{2+}$ .

## Introduction

Given their unique locations, vascular endothelial cells (ECs) appear to be one of the primary targets of hypoxia/reoxygenation (H/R) injury (1,2). The generation of and substantially increased levels of reactive oxygen species (ROS) (3), have been widely implicated in EC injury (4,5). Xanthine oxidoreductase (XOR) exists as two distinct enzyme forms: xanthine dehydrogenase (XDH) and xanthine oxidase (XO). XDH requires  $\text{NAD}^+$  to reduce hypoxanthine to xanthine. XO requires  $\text{O}_2$  for purine oxidation, thereby generating ROS (6). XO has been identified as a source of ROS in atherosclerosis (7), coronary artery disease (8) and heart failure (9). An *in vivo* study showed that XDH expression is increased during H/R in rat kidneys (10). Another *in vitro* study demonstrated that the XDH-to-XO conversion is stimulated by hydrogen peroxide and calcium ( $\text{Ca}^{2+}$ ) in bovine aortic endothelial cells (11). Furthermore, the p38 mitogen-activated protein kinase (p38 MAPK), janus kinase 2 (JAK2) and signal transducers and activators of transcription (STAT) signaling pathways are reportedly involved in the process of XO activation and XDH-to-XO conversion during hypoxia in pulmonary microvascular endothelial cells (12,13).

Hepatocyte growth factor (HGF) has been found to promote survival, proliferation and morphogenesis by activating its receptor cMet. HGF enhances the migration of epithelial cells following acute kidney injury (14). HGF has also been found to regulate neovascularization in developing fat pads (15). In our previous study, we demonstrated that HGF may inhibit XO production and activation by reducing the cytosolic  $\text{Ca}^{2+}$  concentration increased in response to H/R; thus, HGF protects cardiac microvascular endothelial cells (CMECs) from H/R-induced ROS production and H/R-induced cell apoptosis (16). However,

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**Abbreviation:** ECs, endothelial cells; H/R, hypoxia/reoxygenation; HGF, hepatocyte growth factor; XO, xanthine oxidase; CMECs, cardiac microvascular endothelial cells; XDH, xanthine dehydrogenase; ROS, reactive oxygen species; siRNA, small interfering RNA

**Key words:** hepatocyte growth factor, hypoxia/reoxygenation, xanthine oxidase, endothelial cells, janus kinase 2

the signaling mechanisms through which HGF regulates cytosolic  $\text{Ca}^{2+}$  concentrations and XO activation in CMECs under conditions of H/R remain to be elucidated. In the present study, we examined the signaling pathway through which HGF regulates  $\text{Ca}^{2+}$  concentrations and the activation of XO during H/R in primary cultured rat CMECs.

## Materials and methods

**Isolation and culture of CMECs.** A total of 80 five-to-seven day old Sprague-Dawley (SD) rats weighing 12–16 g, were purchased from the Experimental Animal Center of the Chinese PLA General Hospital (Beijing, China). Ethics approval was obtained from the Ethics Committee of the Experimental Animal Center of the Chinese PLA General Hospital (approval no. SCXK20120001). All procedures were performed according to the National Institutes of Health Guideline for the Care and Use of Laboratory Animals. The rats were sacrificed with an overdose of isoflurane and the hearts were removed. The enzyme dissociation method based on the one described by Nishida *et al* was used (17). The cells were collected and resuspended in Dulbecco's modified Eagle's medium (DMEM) (#12100046; Gibco, Grand Island, NY, USA) supplemented with 20% fetal bovine serum (FBS) (#YS-OS-001091; HyClone, Logan, UT, USA) and then seeded in 25 cm<sup>2</sup> polystyrene flasks. Cell purity was identified by morphological (18) and immunohistochemical characteristics (4): the CMEC monolayer displayed a uniform 'cobblestone' morphology and positive immunohistochemical assays of factor VIII (#ab61910; Abcam, Cambridge, MA, USA) and CD31 (#sc71873; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) (>95%).

**H/R procedure and drug treatment.** The H/R procedure was achieved by subjecting the cells to 4 h of hypoxia and 1 h of reoxygenation. For hypoxic exposure, the cells were incubated in D-Hank's solution (in mM: 136.89 NaCl, 5.37 KCl, 4.166  $\text{NaHCO}_3$ , 0.44  $\text{KH}_2\text{PO}_4$ , 0.338  $\text{Na}_2\text{HPO}_4$ , pH 7.3–7.4 at 37°C) saturated with 95%  $\text{N}_2$  and 5%  $\text{CO}_2$ . The pH was adjusted to 6.8 to mimic ischemic conditions. The cells were placed in a hypoxic incubator (Invivo2; Ruskinn Technology Ltd., Pencoed, UK) that was equilibrated with 95%  $\text{N}_2$  and 5%  $\text{CO}_2$ . Ambient  $\text{O}_2$  levels in the incubator were monitored by an  $\text{O}_2$  analyzer (series-2000; Alpha Omega Instruments, Cumberland, RI, USA). Following hypoxic exposure, the culture medium was rapidly replaced with DMEM together with 1% FBS to initiate the reoxygenation procedure (19–21).

For drug treatment, the CMECs were pre-incubated with 30  $\mu\text{M}$  AG490 (#S1509), 30  $\mu\text{M}$  SB203580 (#S1863), 1  $\mu\text{M}$  LY294002 (#S1737) (all from Beyotime Biotech, Jiangsu, China) or 10 or 20 ng/ml HGF (#294-HG-005; R&D Systems, Inc., Minneapolis, MN, USA) for 30 min prior to exposure to hypoxic conditions.

**Measurement of ROS generation.** Following the different treatments, the CMECs were incubated with 2 mM DCFH-DA (#287810; Sigma-Aldrich, St. Louis, MO, USA) for 20 min at 37°C in a 5%  $\text{CO}_2$  incubator. The cells were washed and resuspended in phosphate-buffered saline (PBS) at a concentration of  $1 \times 10^6$  cells/ml. DCF fluorescence was analyzed using a flow cytometer (Becton-Dickinson, Mountainview, CA, USA) at

the excitation and emission wavelengths of 514 and 525 nm, respectively. Untreated cells served as controls. The amount of ROS was calculated as the fold-increase in DCF fluorescence compared with the controls (22).

**Small interfering RNA (siRNA) transfection experiments.** The transient transfection of CMECs with 50 nM siRNA oligonucleotide was performed using Lipofectamine RNAiMAX reagent (#13778030; Invitrogen, Dublin, Ireland). The cells were seeded in 6-well plates ( $2 \times 10^5$  cells/well) for these studies. The following siRNA sequences were used: rat XDH siRNA, 5'-CCACCUCCAAGAUUCAUAUTT-3'; rat phosphoinositide 3-kinase (PI3K) siRNA, 5'-GCAGCCAGCUCUGAUAAUATT-3'; rat JAK2 siRNA, 5'-GCCCCAAGGACUUCAACAATT-3' and rat p38 MAPK siRNA, 5'-GGACCUCUUAUAGACGAATT-3'. These siRNAs and their non-targeting sequences (negative controls) were synthesized by GenePharma Co., Ltd. (Shanghai, China). After 48 h of transfection, the CMECs were subjected to the H/R procedure. Finally, the cells were harvested for other experiments.

**Measurement of cytosolic  $\text{Ca}^{2+}$ .** The CMECs were loaded with fluo-3 (#F23915; Invitrogen, Carlsbad, CA, USA) in 1% working solution at 37°C for 30 min. The cells were washed three times with  $\text{Ca}^{2+}$ -free PBS to remove extracellular fluo-3 AM, and then resuspended in PBS at a concentration of  $1 \times 10^6$  cells/ml. The cells were analyzed by flow cytometry (Becton-Dickinson) at an excitation wavelength of 488 nm, and an emission wavelength of 530 nm (23). The untreated cells served as controls.

**Western blot analysis.** The CMECs were homogenized in RIPA lysis buffer (#P0013C; Beyotime) containing 1X Phosphatase Inhibitor Cocktail (#5870S; Cell Signaling Technology, Beverly, MA, USA) and 1  $\mu\text{g}/\text{ml}$  each of aprotinin (A1153; Sigma-Aldrich) and leupeptin (#L2884; Sigma-Aldrich). Forty micrograms of protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membranes, and then probed with antibodies for XO (#ab109235; Abcam), phospho-PI3 kinase p85 (Tyr458)/p55 (Tyr199) (#4228), PI3 kinase p85 (19H8) (#4257), phospho-p38 MAP kinase (Thr180/Thr182) (#4631), p38 MAPK (D13E1) (#8690), JAK2 (D2E12) (#3230) and phospho-JAK2 (Tyr1007/1008) (C80C3) (#3776) (all from Cell Signaling Technology, Beverly, MA, USA). The same membranes were reprobed with an antibody for tubulin (#AT819; Beyotime). The blotting film was quantified using a scanner and a densitometry program (ImageJ; <https://imagej.nih.gov/ij/index.html>) (24). To quantify the phospho-specific signal in the activated samples, the background was subtracted and the band was normalized to the amount of tubulin or total target protein in the lysate.

**Statistical analysis.** Statistical comparisons were performed using the paired, two-tailed Student's t-test for experiments consisting of two groups only, with one-way ANOVA and a multiple comparison method for experiments consisting of more than two groups. A  $p < 0.05$  was considered to indicate a statistically significant difference. Data are presented as the means  $\pm$  SE.

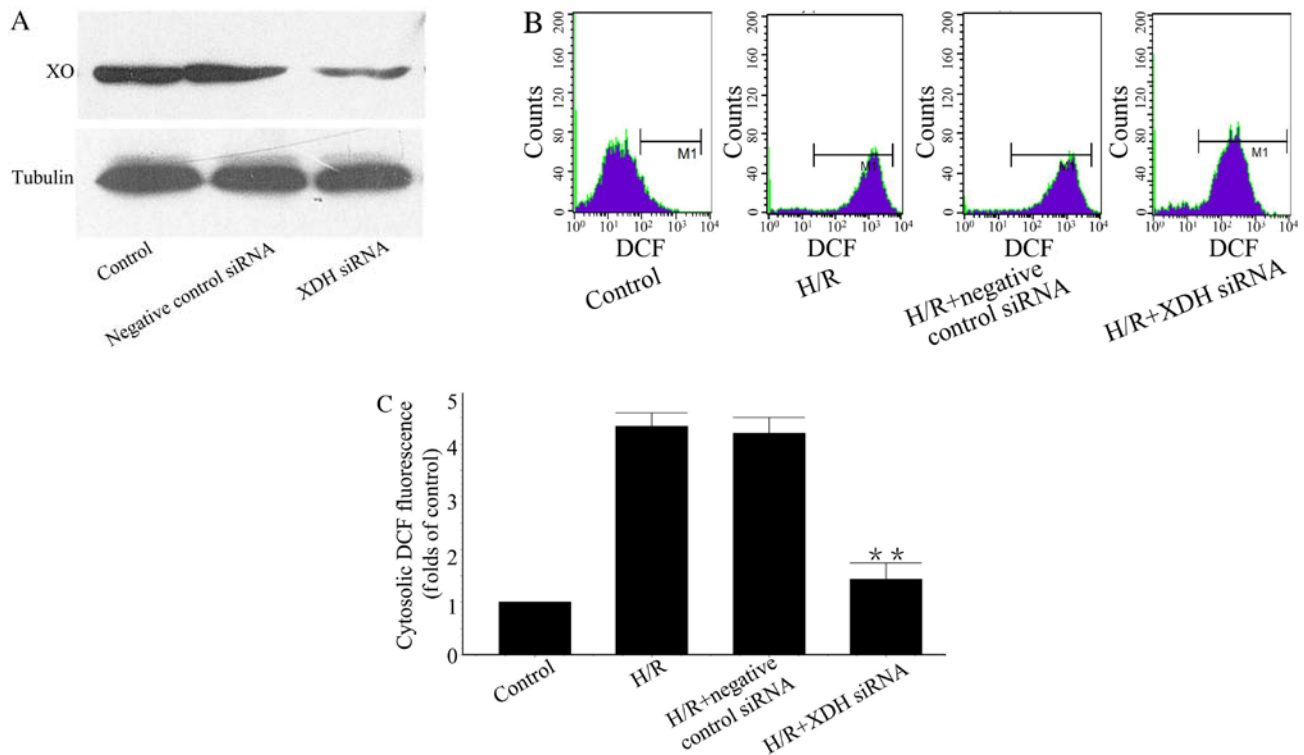


Figure 1. Knockdown of xanthine oxidase (XO) attenuates the hypoxia/reoxygenation (H/R)-induced production of ROS. (A) Representative western blot of XO and tubulin expression in cardiac microvascular endothelial cells (CMECs) transfected with either no siRNA, negative control siRNA or xanthine dehydrogenase (XDH) siRNA. (B) Representative data for flow cytometric analysis of DCFH-DA-stained CMECs transfected with negative control siRNA or XDH siRNA, and compared with the H/R group. (C) Summary data (n=3 biological replicates) of flow cytometric analysis of DCFH-DA-stained CMECs under conditions of H/R transfected with negative control siRNA or XDH siRNA, and compared with the H/R group. \*\*p<0.01 vs. H/R group.

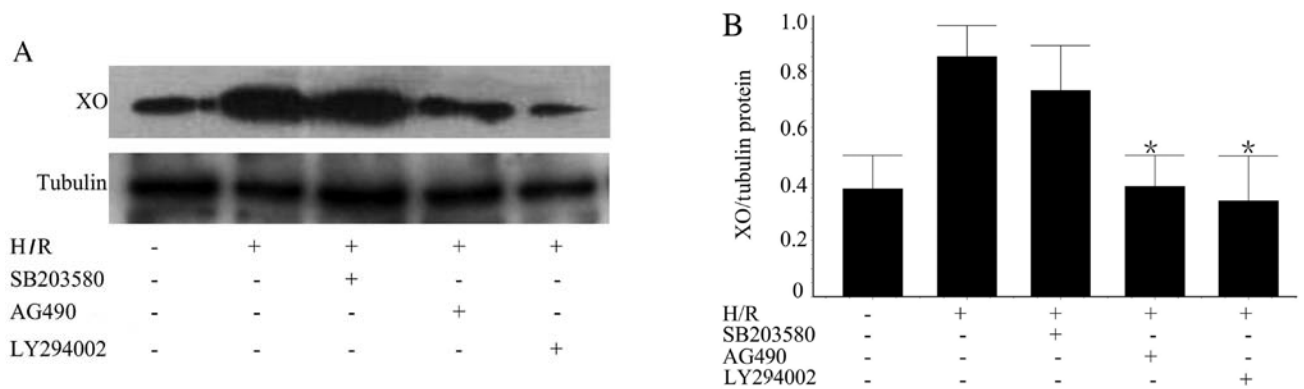


Figure 2. PI3K and JAK2 signaling pathways are involved in the production and activation of xanthine oxidase (XO). (A) Representative western blot of XO and tubulin expression in cardiac microvascular endothelial cells (CMECs) following hypoxia/reoxygenation (H/R). LY294002 and AG490 significantly decreased the increase in the XO protein level induced by H/R. (B) Summary data (n=3 biological replicates) of western blot analysis of XO and tubulin expression in CMECs following H/R. LY294002, SB203580 and AG490 treatment group compared with H/R group. \*p<0.05 vs. H/R group.

## Results

*XO plays a key role in the H/R-induced production of ROS.* In our previous study, the production of ROS following H/R was significantly attenuated by allopurinol (20 and 40  $\mu$ mol/l), an inhibitor of XO (16). In the present study, the expression of XO was knocked down by XDH siRNA (Fig. 1A). Four hours of hypoxia increased intracellular DCF fluorescence compared with normoxia (control group). The transfection of XDH siRNA attenuated the increased production of ROS following H/R (Fig. 1B and C).

*PI3K and JAK2 pathways are involved in the production and activation of XO.* To determine whether PI3K, p38 MAPK or JAK2 signaling pathways are involved in the production and activation of XO, we examined the effect of PI3K inhibitor LY294002, p38 MAPK inhibitor SB203580 and JAK2 inhibitor AG490 on the production and activation of XO. Pre-treatment with LY294002 and AG490 inhibited H/R-mediated XO production (Fig. 2A and B). The phosphorylation of PI3K and JAK2 was significantly increased following H/R as compared with the normoxia controls (Fig. 2C and D). However, the phosphorylation of p38 MAPK was not found to be increased

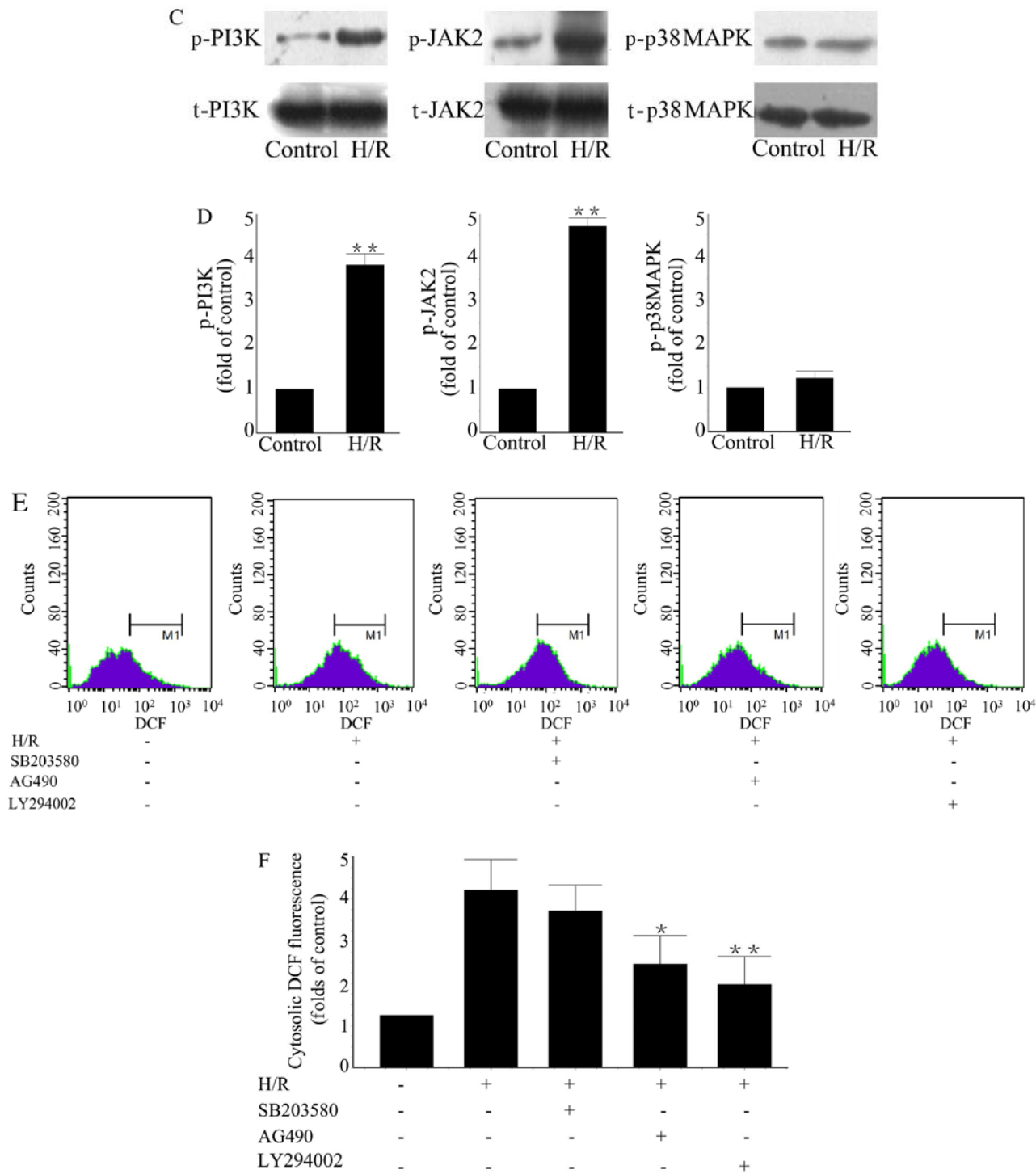


Figure 2. Continued. PI3K and JAK2 signaling pathways are involved in the production and activation of xanthine oxidase (XO). (C) Representative western blots of phosphorylated (p-)PI3K, total (t-)PI3K, p-JAK2, t-JAK2, p-p38 MAPK and t-p38 MAPK expression in cardiac microvascular endothelial cells (CMECs) following hypoxia/reoxygenation (H/R). H/R activates the PI3K and JAK2 signaling pathways but not the p38 MAPK signaling pathway. (D) Summary data (n=3 biological replicates) of western blot analysis of p-PI3K, t-PI3K, p-JAK2, t-JAK2, p-p38 MAPK and t-p38 MAPK expression in CMECs. \*\*p<0.01 vs. control group. (E) Representative data for flow cytometric analysis of DCFH-DA-stained CMECs following H/R. LY294002, SB203580 and AG490 treatment groups compared with H/R group. (F) Summary data (n=3 biological replicates) of flow cytometric analysis of DCFH-DA-stained CMECs following H/R. LY294002 (1  $\mu$ M), SB203580 (30  $\mu$ M) and AG490 (30  $\mu$ M) treatment groups compared with H/R group. \*p<0.05 vs. H/R group; \*\*p<0.01 vs. H/R group.

after H/R (Fig. 2C and D). ROS production following H/R was also partly blocked by LY294002 and AG490, respectively (Fig. 2E and F). These data indicated that XO activation induced by H/R in CMECs is mediated through the PI3K and JAK2 signaling pathways rather than the p38 MAPK signaling pathway.

*PI3K siRNA and JAK2 siRNA downregulate the production and activation of XO.* To further confirm the involvement of PI3K and JAK2 signaling pathways in the production and activation of XO following H/R, CMECs were transfected with either PI3K siRNA, JAK2 siRNA or p38 MAPK siRNA to introduce knockdown (Fig. 3A). When the expression of

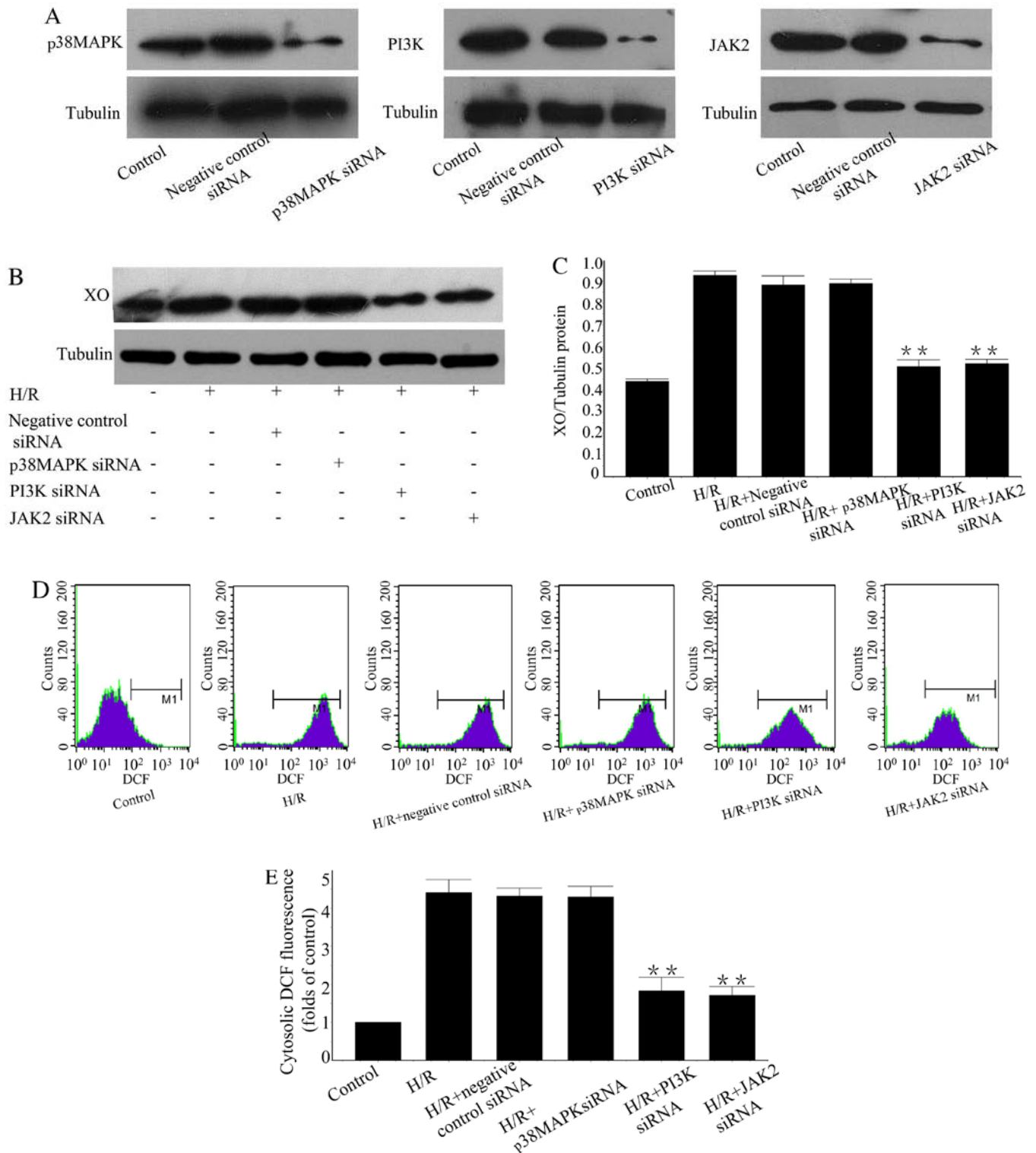


Figure 3. PI3K siRNA and JAK2 siRNA downregulate the production and activation of xanthine oxidase (XO). (A) Representative western blots of p38 MAPK, PI3K, JAK2 and tubulin expression in cardiac microvascular endothelial cells (CMECs). (B) Representative western blot of XO and tubulin expression in CMECs following hypoxia/reoxygenation (H/R). PI3K siRNA-, p38 MAPK siRNA-, JAK2 siRNA- and negative control siRNA-transfected groups compared with H/R group. (C) Summary data (n=3 biological replicates) for western blot analysis of XO and tubulin expression in CMECs following H/R. PI3K siRNA-, p38 MAPK siRNA-, JAK2 siRNA- or negative control siRNA-transfected groups compared with H/R group. \*\*p<0.01 vs. H/R group. (D) Representative flow cytometric analysis of DCFH-DA-stained CMECs after H/R. PI3K siRNA-, p38 MAPK siRNA-, JAK2 siRNA- or negative control siRNA-transfected groups compared with H/R group. (E) Summary data (n=3 biological replicates) for flow cytometric analysis of DCFH-DA-stained CMECs after H/R. PI3K siRNA-, p38 MAPK siRNA-, JAK2 siRNA- or negative control siRNA-transfected groups compared with H/R group. \*\*p<0.01 vs. H/R group.

PI3K and JAK2 was inhibited by their respective siRNAs, the H/R-induced increase in XO production was downregulated (Fig. 3B and C). ROS production following H/R was

also partly blocked by PI3K siRNA and JAK2 siRNA, respectively (Fig. 3D and E) However, p38 MAPK siRNA did not exert similar effects. These data further confirmed that the

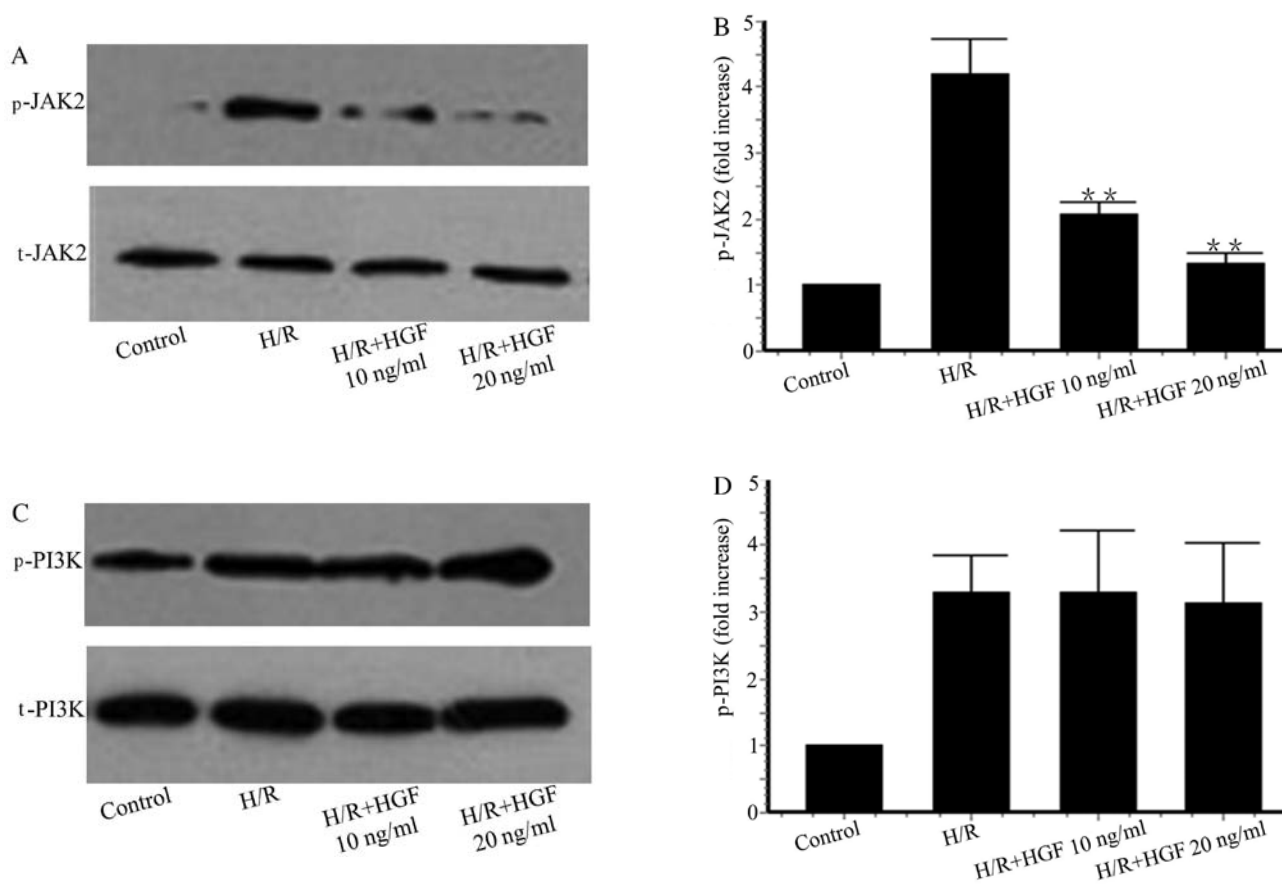


Figure 4. Hepatocyte growth factor (HGF) inhibits activation of the JAK2 signaling pathway but has no effect on the PI3K signaling pathway. (A) Representative western blot of phosphorylated (p-)JAK2 and total (t-)JAK2 expression in cardiac microvascular endothelial cells (CMECs) following hypoxia/reoxygenation (H/R). HGF significantly decreased the H/R-induced activation of the JAK2 pathway. (B) Summary data (n=3 biological replicates) for western blot analysis of p-JAK2 and t-JAK2 in CMECs after H/R. HGF (10 and 20 ng/ml) treatment groups compared with H/R group. \*\*p<0.01 vs. H/R group. (C) Representative western blot of p-PI3K and t-PI3K expression in CMECs after H/R. HGF had no effect on the PI3K signaling pathway. (D) Summary data (n=3 biological replicates) for western blot analysis of p-PI3K and t-PI3K expression in CMECs following H/R.

production and activation of XO induced by H/R in CMECs is mediated through the PI3K and JAK2 signaling pathways rather than the p38 MAPK signaling pathway.

**HGF inhibits JAK2 activation but not PI3K activation.** The phosphorylation of JAK2 and PI3K was evaluated by western blot analysis in CMECs pre-treated with HGF (10 and 20 ng/ml). The phosphorylation of JAK2 induced by H/R was inhibited by HGF (Fig. 4A and B). However, the phosphorylation of PI3K induced by H/R was unaffected by HGF (Fig. 4C and D). These findings suggest that HGF inhibited the activation and production of XO through the JAK2 signaling pathway.

**JAK2 siRNA downregulates the concentration of cytosolic calcium.** In our previous study, we found that HGF inhibits XO activation by reducing cytosolic  $Ca^{2+}$  concentrations induced by H/R (16). We further studied whether the JAK2 signaling pathway regulates cytosolic  $Ca^{2+}$  concentrations. JAK2 knockdown was achieved by JAK2 siRNA, and resulted in a reduction in the cytosolic  $Ca^{2+}$  concentration induced by H/R (Fig. 5). Taken together, these findings suggest that HGF reduced cytosolic  $Ca^{2+}$  concentrations by inhibiting JAK2 phosphorylation.

## Discussion

Excessive oxidative stress is believed to be an important contributor to H/R injury. In our previous study, we revealed that HGF protects CMECs from H/R-induced apoptosis by reducing ROS production (16). The findings of the present study indicate that H/R increased DCF oxidation. DCF detects  $H_2O_2$  but does not detect superoxide (25). However,  $H_2O_2$  accounts for 90% of ROS production under hypoxic conditions and superoxide accounts for 10% (26). DCF oxidation appears to be a reliable method for the detection of cellular ROS production (25,27). Potential sources of ROS include XO (6), NADPH oxidase (1), the mitochondrial respiratory chain (21), and the metabolic cascade of arachidonic acid (28). XO is the major source of ROS in the rat jugular venous (29) and rat pulmonary circulation (27) following H/R. The mitochondrial respiratory chain is the major source of ROS in embryonic chick cardiomyocytes (21) and human umbilical vein ECs (HUVECs) (30). Thus, we hypothesized that the major source of ROS following H/R is both species-specific and organ-specific. When XOR expression was knocked down by XDH siRNA, H/R-induced ROS production in CMECs was also attenuated. XO accounts for, at least part of, the ROS production induced by H/R in CMECs.



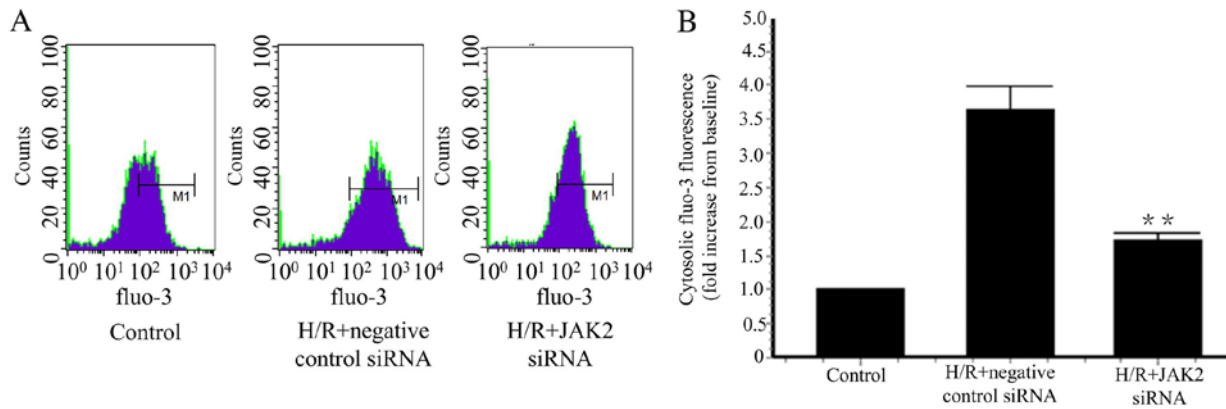


Figure 5. JAK2 siRNA downregulates cytosolic calcium concentrations. (A) Representative data for flow cytometric analysis of fluo-3-stained cardiac microvascular endothelial cells (CMECs) following hypoxia/reoxygenation (H/R). JAK2 siRNA- or negative control siRNA-transfected groups compared with H/R group. (B) Summary data (n=3 biological replicates) for flow cytometric analysis of fluo-3-stained CMECs after H/R. JAK2 siRNA- or negative control siRNA-transfected group compared with H/R group. \*\*p<0.01 vs. H/R group.

The signaling pathways involved in the production and activation of XO are controversial under different circumstances in different cell types. p38 MAPK and CK2 have been found to be involved in the activation of XO following hypoxia in rat pulmonary microvascular ECs (RPMECs) (12). JAKs and STATs are involved in the hypoxia-mediated activation of XO in lung microvascular ECs (LMVECs) (13). The phosphorylation of PI3K increases ROS production during hypoxia in endothelial progenitor cells (31) and in mouse pulmonary microvascular ECs (PMVECs) (25). In the present study, AG490 and LY294002 partially blocked the increase in ROS production following H/R. The PI3K and JAK2 signaling pathway is significantly activated after H/R whereas the p38 MAPK signaling pathway is unaffected. The pre-treatment of CMECs with AG490 and LY294002 markedly attenuated XO protein levels. The pre-treatment of CMECs with SB203580 did not have the above-mentioned effect. Furthermore, when the knockdown of JAK2 or PI3K was achieved by siRNA, increases in the XO protein levels and ROS production were greatly attenuated. These data show that the PI3K and JAK2 signaling pathways are involved in the upregulation and activation of XO following H/R. However, HGF inhibits the activation of the JAK2 signaling pathway but not the PI3K signaling pathway. In our previous study, we reported that HGF inhibits the activation and production of XO by reducing cytosolic  $\text{Ca}^{2+}$  concentrations in CMECs after H/R (16). Thus, HGF inhibits XO activation by inhibiting JAK2 signal pathway. In the present study, JAK2 knockdown by JAK2 siRNA significantly reduced cytosolic  $\text{Ca}^{2+}$  concentrations. This finding is in agreement with the results of a previous study which demonstrated that AG490, the JAK2 signal inhibitor, blocked an  $\text{H}_2\text{O}_2$ -induced increase in intracellular  $\text{Ca}^{2+}$  in U937 cells (32). It has been recognised that XO activation is regulated by cytosolic  $\text{Ca}^{2+}$ , and an unidentified  $\text{Ca}^{2+}$ -dependent protease is involved in the cleavage of XDH to XO (11). A heat-labile protease that cleaves XDH to XO has also been found in the mitochondrial intermembrane space (33). In our previous study, when CMECs were pre-treated with BAPTA-AM, a cell permeable calcium chelator, the H/R-induced activation of XO was blocked. HGF prevents

JAK2 activation, reduces cytosolic  $\text{Ca}^{2+}$  concentrations and in turn, inhibits XO activation in CMECs following H/R.

In conclusion, these findings suggest a novel mechanism whereby HGF regulates H/R-induced XO activation in CMECs. The upregulation and activation of XO as well as increased ROS production following H/R primarily involve the PI3K and JAK2 signaling pathways but not the p38 MAPK signaling pathway. HGF inhibits the activation of JAK2. The knockdown of JAK2 attenuated cytosolic  $\text{Ca}^{2+}$  concentrations in CMECs following H/R. Thus, HGF inhibits XO activation by inhibiting JAK2 activation and reducing cytosolic  $\text{Ca}^{2+}$  concentrations in CMECs following H/R. HGF may exhibit protective and therapeutic effects against H/R injury in H/R-related diseases.

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