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RESEARCH ARTICLE

Effects of Empagliflozin on Intermittent Hypoxia-Induced TRAF3IP2-Dependent Human Aortic Smooth Muscle Cell Proliferation

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ABSTRACT

Aims: Chronic intermittent hypoxia (IH), a characteristic feature of obstructive sleep apnea (OSA), contributes to cardiovascular diseases, including atherosclerosis, potentially through persistent oxidative stress and inflammation. TRAF3IP2 (TRAF3 Interacting Protein 2) is an oxidative stress-responsive proinflammatory adapter molecule and plays a causal role in a preclinical model of atherosclerosis. Since SGLT2 (Sodium/Glucose Cotransporter 2) inhibitors have shown protective effects in CVD by inhibiting oxidative stress and inflammation, we hypothesized that IH promotes the crosstalk between oxidative stress and TRAF3IP2, resulting in IL-6-dependent human aortic smooth muscle cell (SMC) proliferation, and that these effects are inhibited by the SGLT2 inhibitor empagliflozin.

Materials and methods: Primary human aortic SMC were exposed to various cycles of IH. Normoxia served as a control. To understand the molecular mechanisms underlying IH-induced nitroxidative stress, TRAF3IP2 and IL-6 induction, and SMC proliferation and those targeted by empagliflozin were determined by treating SMC with various pharmacological inhibitors and viral vectors.

Results: IH upregulated TRAF3IP2 expression, TRAF3IP2-dependent superoxide, hydrogen peroxide and nitric oxide generation, NF-κB and HIF-1α activation, IL-6 induction, and SMC proliferation. Exposure to IL-6 by itself induced SMC proliferation in part via TRAF3IP2, IL-6R, gp130, JAK, and STAT3. Further, SMC express SGLT2 at basal conditions, and is upregulated by both IH and IL-6. Importantly, empagliflozin inhibited IH-induced TRAF3IP2 upregulation, reactive oxygen and nitrogen species generation, TRAF3IP2-dependent HIF-1α and NF-κB activation, IL-6 induction, and IL-6-dependent JAK-STAT3-mediated SMC proliferation. Moreover, empagliflozin inhibited IL-6-induced STAT3-dependent SMC proliferation.

Conclusions: These results suggest the therapeutic potential of empagliflozin in IH and inflammatory vascular proliferative diseases associated with OSA.

Keywords: SGLT2, inflammation, oxidative stress, nitric oxide, intermittent hypoxia

1. Introduction

Obstructive sleep apnea (OSA), a chronic sleep-associated breathing disorder, affects more than 20 million people in the USA [1]. OSA often results in intermittent hypoxia (IH) consequent to cyclical apneas and hypopneas,[2] resulting in persistent oxidative stress and inflammation in various organs, including the heart and blood vessels. In cultured rat aortic smooth muscle cells (SMC), IH, but not normoxia (Nx) or sustained hypoxia, stimulates proliferation, in part via increased expression of erbB2 receptor and the members of the EGF family [3]. In addition to its mitogenic effects, IH has also been shown to induce SMC hypertrophy, resulting in increased intima-media thickness and adverse vascular remodeling in preclinical models [4]. Independent of *in vitro* or *in vivo* setting, IH increases oxidative stress and inflammation, and could contribute t vascular remodeling.

We have previously demonstrated that the cytoplasmic adapter molecule TRAF3IP2 (TRAF3 Interacting Protein 2) [5,6] not only plays a role in oxidative stress, but is also a potent inducer of inflammatory cytokines and chemokine expression both *in vitro* and *in vivo*. [7,8] For example, IL-17, a proinflammatory cytokine, [5,6,9] induces SMC migration and proliferation via TRAF3IP2-dependent JNK, p38 MAPK, AP-1, NF-kB and CREB activation [10]. Importantly, TRAF3IP2 deletion in Apo*E* knockout mice significantly reduces western diet-induced total aortic plaque area, plaque necrotic area, and the induction of multiple inflammatory mediators, including TNFa, CXCL1, ICAM-1, and VCAM1 in female mice, [11] indicating that TRAF3IP2 plays a causal role in oxidative stress and inflammation in a disease model characterized by increased SMC proliferation. Because IH induces oxidative stress and inflammation, and as TRAF3IP2 mediates both processes, we hypothesized that targeting TRAF3IP2 blunts IH-induced oxidative stress, inflammation, and SMC proliferation.

SGLT2 (Sodium/Glucose Cotransporter 2) inhibitors are a novel class of blood sugar lowering drugs used increasingly in type 2 diabetics [12]. In fact, several landmark clinical trials proved its beneficial effect in diabetic subjects with high cardiovascular risk. For example, in the EMPA-REG OUTCOME trial, administration of empagliflozin along with the standard care, markedly reduced cardiovascular outcomes and mortality in type II diabetic patients. In the EMPEROR-reduced clinical trial, empagliflozin along with the recommended heart failure therapy, had markedly reduced the risk of cardiovascular death or hospitalization for heart failure irrespective of the diabetic state [13]. Similarly, in the EMPEROR-Preserved clinical trial, empagliflozin reduced the combined risk of cardiovascular death or hospitalization of subjects diagnosed with HFpEF, independent of the diabetic state [14]. These successful clinical trials subsequently led to multiple experimental investigations that focused on investigating the anti-inflammatory and anti-oxidative effects of empagliflozin in various cell types.

We recently reported that empagliflozin targets hyperglycemia-induced TRAF3IP2 expression, oxidative stress, and inflammation,epithelial-to-mesenchymal transition, and migration of cultured proximal tubule epithelial cells [15]. We have also demonstrated the vascular protective effects of empagliflozin in diabetes-prone db/db mice, where it decreased aortic stiffness [16]. Arterial stiffness contributes to atherosclerosis development and incident cardiovascular events [17]. Recently, we and others have reported that canagliflozin and empagliflozin inhibit vascular SMC proliferation under normal glucose conditions without inducing cell death [18,19]. We demonstrated that empagliflozin inhibits IL-17A-mediated TRAF3IP2/NLRP3/Caspase-1-dependent IL-1b and IL-18 secretion. [19] Since SGLT2 inhibitors exert pleiotropic antioxidant and anti-inflammatory effects independent of their glucose lowering properties, we hypothesized that empagliflozin will inhibit IH-induced oxidative stress, inflammation, and SMC proliferation by targeting TRAF3IP2 and its downstream signaling intermediates.

Confirming our hypotheses, treatment with the SGLT2 inhibitor empagliflozin inhibited IH-induced TRAF3IP2 upregulation, reactive oxygen and nitrogen species generation, TRAF3IP2-dependent HIF-1α and NF-κB activation, IL-6 induction, and IL-6-dependent JAK-STAT3-mediated SMC proliferation. These results suggest that empagliflozin has therapeutic potential in IH-associated chronic inflammatory and vascular proliferative diseases.

2. Materials and methods

Materials

Empagliflozin (#S8022; 1 μM in DMSO for 15 min), the JAK inhibitor Tofacitinib (#S5001), the STAT inhibitor HO-3867 (S7501), and the gp130 inhibitor SC144 (#S7124; 2 μM in DMSO for 1 h) [20] were all purchased from Selleck Chemicals (Houston, TX). The iNOS inhibitor AMT [21] was purchased from Tocris (Minneapolis, MN) and used at a concentration of 100 μM in water for 30 min. The iNOS-specific inhibitor, 1400W, was purchased from Tocris, and used at a concentration of 1μM in water for 1 h. N-acetyl-L-cysteine (NAC, 5 mM in water for 30 min; #A7250), Polymyxin B sulphate salt (10 μg/ml in water for 2 h; #P4932), and other biochemicals were purchased from Millipore-Sigma (St. Louis, MO, USA). The peptide inhibitor of Nox2 assembly, gp91 ds-tat (#AS-63818) and its scrambled peptide (#AS-63855) were purchased from AnaSpec (Fremont, CA), and used at a concentration of 1 μM for 1 h prior to IH as previously described [19]. The Nox1/4 inhibitor GKT137831 (#17164; 5 μM in DMSO for 15 min prior to IH) was purchased from Cayman Chemical (Ann Arbor, MI) and used as before [22]. DMSO was purchased from EMD

Biosciences (San Diego, CA). Pierce™ BCA Protein Assay Kit (#23227), SuperSignal® West Femto Maximum Sensitivity Substrate (#34096), Pierce™ Protein MW marker and Pierce™ Chromogenic Endotoxin Quant Kit (#A39552S) were purchased from ThermoFisherScientific (Waltham, MA). In initial experiments, SMC were treated with empagliflozin at concentrations ranging between 0.1 to 5 μM for 15 min prior to IH. Since significant inhibitory effects were observed at 1μM (data not shown), this concentration was used in all subsequent experiments. In fact, pharmacokinetics revealed its C_{max} to be between 0.5 and 1.11 μM at 25 and 50 mg dose, respectively [23]. Bioactive recombinant human (rh) IL-6 protein (#206-IL) was purchased from R&D Systems (Minneapolis, MN). The recombinant IL-6 protein contained trace amount of endotoxin (<0.10 enzyme units/μg) as determined by the Limulus Amebocyte Assay (R&D Systems). Normal adult human kidney whole tissue lysate (#NB820–59231) was purchased from Novus Biologicals (Centennial, CO)[19]. LDH (Lactate Dehydrogenase) Cytotoxicity Assay Kit (#601170) was purchased from Cayman Chemical, (Ann Arbor, MI). NE-PER™ Nuclear and cytoplasmic extraction reagents (#78835) and CyQUANT™ Cell Proliferation Assay (#C7026) were purchased from ThermoFisherScientific. Vybrant® MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) Cell Proliferation Assay Kit (V13154) was purchased from Cayman Chemical (Ann Arbor, MI).

Cell culture and intermittent hypoxia

Human aortic SMC, purchased from LONZA (#CC-2571), were grown in SmGM-2 basal medium with SmGM™–2 SingleQuots™ supplements (LONZA, #CC-4149) as previously described [19]. These primary cells, obtained from a single donor (11-year-old female, non-diabetic and non-smoker) and a single lot (18TL117408), were authenticated by the supplier for being positive for αSMA (α smooth muscle actin) and SM-MHC (smooth muscle myosin heavy chain), but not for VWF (Von Willebrand Factor). Moreover, the cells were negative for mycoplasma contamination. Cells were pooled from three aliquots and used in experiments. At 70–80% confluency, culture medium was replaced with basal medium containing 0.5% bovine serum albumin (conditioning medium). After 48 h incubation, SMC underwent IH as previously described [24]. In brief, SMC were exposed to IH (alternating cycles of 1.5% O_2 for 30 sec followed by 20% O_2 for 5 min at 37°C) as indicated. Normoxia served as a control; cells were exposed to normoxia for a duration similar to the number of IH cycles. For example, normoxia (Nx) for 10 cycles is equivalent to 5 min and 30 sec x10= 53 min. Ambient O_2 levels in the IH chamber were analyzed using the O_2 analyzer from Alpha Omega Instruments Corp (Houston, TX). HK-2 (human kidney-2) cells were purchased from ATCC® (#CRL-2190 Manassas, VA). HK-2 is an immortalized human proximal tubule cell line derived from a single cell isolated from an adult male normal kidney. Cells were authenticated by ATCC®. The cells were cultured in DMEM/F12 medium as previously described [19].

Adenoviral and lentiviral transduction

Adenoviral vector expressing short hairpin RNA (shRNA) targeting human TRAF3IP2 (Ad.TRAF3IP2 shRNA) was previously described [19]. Ad-GFP-U6-shRNA, used as a control, was purchased from Vector Biolabs (#1122). For adenoviral transduction, SMCs were grown to 60–70% confluency in complete medium. The medium was replaced with PBS, and the cells were infected at multiplicity of infection (moi)10 for 1 h at 22°C. The infection medium was replaced with medium containing 0.5% BSA. After 24 h, the cells were exposed to IH or IL-6 as indicated. Knockdown of TRAF3IP2 was confirmed by Western blotting. MyD88 served as a non-targeting control. Tubulin served as a loading control. Lentiviral shRNA against NF-κBp65 (TRC#0000014684), HIF-1α (TRC#0000003810), IL-6R (TRC#0000058778); gp130 (TRC#0000058284), and SGLT2 (TRC# 0000043603) were purchased from Millipore-Sigma. For lentiviral transduction, SMCs at 50–60% confluency were infected with the indicated shRNA at a multiplicity of infection (moi) of 0.5 for 48 h in complete media. Infection with viral vectors had no significant effect on SMC viability or adherence to culture dishes.

Superoxide, H_2O_2 and nitric oxide production

The generation of superoxide and H_2O_2 was measured following 50 cycles of IH. Normoxia (Nx) served as a control. Superoxide generation was quantified by the lucigenin-enhanced chemiluminescence assay as previously described [19]. H_2O_2 production was measured according to the manufacturer's instructions using a commercially available kit in the presence of horseradish peroxidase (0.1 unit/ml, Amplex Red: and 50 μM) as described in our previous report [19]. Nitrite production in culture supernatant was quantified 24 hours after IH by the Griess Reaction (Cayman Chemical Company; Cat#: 780001).

mRNA expression

IL-6 mRNA expression was analyzed by RT-qPCR [7,8,10,11,19] using Applied Biosystems™ TaqMan™ probes: IL6 (Hs00174131-m1). GAPDH (Hs02786624_g1) served a loading control. All data were normalized to corresponding GAPDH levels, analyzed using $2^{-\Delta\Delta Ct}$ method, and presented as fold change from untreated control, which was considered as 1.

Western blotting

Western blotting was performed as previously described [7,8,10,11,15]. Tubulin served as a loading control. The following primary antibodies were used: TRAF3IP2 (#NB100–56740, Novus Biologicals, Centennial, CO), Tubulin (#2144, Cell Signaling Technology, Inc, Danvers, MA; CST), p-p65 (#3033, CST), Lamin A/C (#4777, CST), p-STAT3 (CST), STAT3 (#9132, CST), cleaved caspase-3 (#ab32040, abcam, Waltham, MA), caspase-3 (#ab90347, abcam), HIF-1α (#ab179483, abcam), IL-6 (#ab233706, abcam), gp130 (#ab217671, abcam), IL-6R (#AF-228-NA, R & D Systems), SGLT2 (#sc-393350, Santa Cruz Biotechnology, Inc., Dallas, TX; SCB) and MyD88 (#sc-74532, SCB). Phospho-p65 (Ser^{536}) was analyzed in equal amounts of nuclear protein extracts isolated from SMC exposed to indicated treatments using NE-PER™ Nuclear and cytoplasmic extraction reagents from ThermoFisherScientific. Lamin A/C served as a loading control. HIF-1α and STAT3 were analyzed in cleared whole cell lysates.

SMC proliferation and viability

SMC proliferation was analyzed as described previously [19,25]. To determine whether IH, pharmacological inhibitors, and viral vectors alter viability, cell viability was analyzed by two independent methods: (i) A cell viability assay was performed according to manufacturer's instructions using the LDH-Glo™ Cytotoxicity Assay (#J2380) from Promega that quantifies LDH release into culture supernatants by SMC exposed to different experimental conditions. As a positive control, maximum LDH release was determined by treating SMC exposed to Nx with 2µl of 10% Triton X-100 per 100µl for 15 minutes before collecting the samples for LDH detection. The results obtained with TritonX-100 treatment was set to 100% LDH release. (ii) Since activation of the effector caspase-3 plays a central role in cell death, we also assessed cell viability by analyzing cleaved caspase-3 and total caspase-3 levels at 12 h by Western blotting using cleared whole cell lysates. H_2O_2 (100 µM) served as a positive control.

Statistical analysis

Values are shown as means ± standard error of mean (SEM). Statistical significance was determined by one-way ANOVA followed by Tukey's post hoc test (GraphPad Prism software, San Diego, CA). Differences are considered significant if the P value is <0.05. Further, though a representative Western blot is shown in the main figures, changes in target protein expression from three to four independent experiments were semi-quantified by densitometry and presented at the bottom or side of respective panels as fold changes over control, which was set at a value of 1. The numbers at the bottom of each panel in figures denote lane numbers.

3. Results

IH, TRAF3IP2, and nitroxidative stress, and SMC proliferation

SMC were exposed to various cycles of IH. The results show that IH increased TRAF3IP2 protein expression progressively with each cycle number, reaching significance after 30 cycles, and peak levels after 50 cycles (Fig. 1A). Its expression did not further increase as the cycle numbers increased to 60. Therefore, in all subsequent experiments, SMC were exposed to 50 cycles of IH. Nx equivalent to 50 cycles of IH showed low TRAF3IP2 expression (Fig. 1A, lane 1). Further, IH increased superoxide generation (Fig. 1B), an effect that was significantly attenuated by TRAF3IP2 knockdown or pretreatment with NAC or gp91 ds-tat. IH also increased H_2O_2 production, and this effect was inhibited by TRAF3IP2 knockdown, NAC or GKT137831 (Fig. 1C; knockdown of TRAF3IP2 was confirmed by Western blotting as shown on the right). IH also increased cumulative nitrite production, an effect that was inhibited by TRAF3IP2 knockdown, NAC or the iNOS inhibitors 1400W and AMT (Fig. 1D). Interestingly, the inhibitors of superoxide, H_2O_2, and nitrite production also inhibited IH-induced TRAF3IP2 expression (Fig. 1E). More importantly, IH stimulated SMC proliferation, an effect that was attenuated by TRAF3IP2 knockdown or inhibitors of superoxide, H_2O_2, and nitrite production (Fig. 1F). However, neither TRAF3IP2 knockdown nor inhibitors of superoxide, H_2O_2, and nitrite production negatively impacted cell viability as evidenced by the low levels of released LDH and caspase-3 activation (Fig. 1G). Together, these results indicate that IH induces SMC proliferation via TRAF3IP2 and nitroxidative stress (Fig. 1).

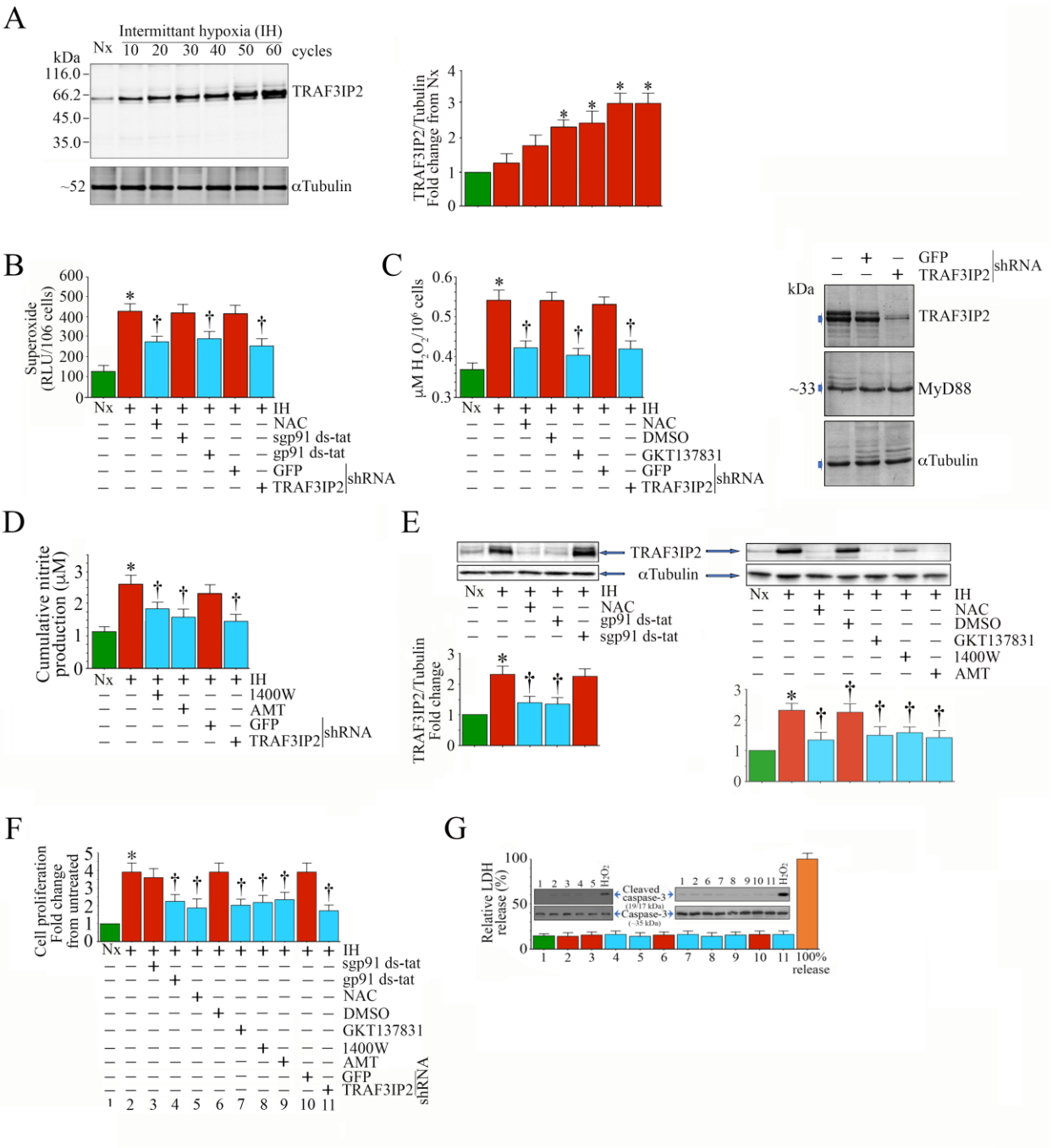

Figure 1. IH induces human aortic SMC proliferation via a crosstalk between TRAF3IP2 and nitroxidative stress. A, IH upregulates TRAF3IP2 expression. At 70-80% confluency, SMC were made quiescent, and exposed to IH for the indicated number of cycles. Nx represents time equivalent to 50 cycles of IH. TRAF3IP2 expression was analyzed by Western blotting using 20 μg of cleared whole cell lysates (n=3). Tubulin served as a loading control. B, IH stimulates superoxide generation via TRAF3IP2 and Nox2. Quiescent SMC exposed to 50 cycles of IH were analyzed for superoxide generation by the lucigenin-enhanced chemiluminescence assay. In a subset of experiments, SMC were exposed to NAC or gp91 ds-tat or transduced with Ad.TRAF3IP2-shRNA prior to IH. sgp91 ds-tat or GFP shRNA served as controls (n=6). Knockdown of TRAF3IP2 was confirmed by Western blotting as shown on the right. The adapter molecule MyD88 served as an off target. C, IH stimulated H_2O_2 production via TRAF3IP2 and Nox4. SMC were treated as in *C*, but with GKT137831 or NAC or transduced with Ad.TRAF3IP2-shRNA, and then analyzed for H_2O_2 production by Amplex Red assay (n=6). D, IH stimulated nitric oxide generation. Quiescent SMC exposed to IH as in *C*, but pretreated with 1400W or AMT, or transduced with Ad.TRAF3IP2-shRNA were analyzed for nitric oxide generation by the Greiss reaction, and the data were presented as cumulative nitrite production in μM (n=6). E, IH upregulates TRAF3IP2 expression via nitroxidative stress. Quiescent SMC treated as in *C*, *D* and *E* were analyzed

for TRAF3IP2 expression by Western blotting (n=3). F, IH stimulates SMC proliferation via TRAF3IP2 and nitroxidative stress. Quiescent SMC treated as in *C-E* were analyzed for proliferation by the CyQUANT™ assay after 48h (n=6) G. The pharmacological inhibitors and the shRNA used did not compromise cell viability as analyzed by a colorimetric LDH release assay and activation of caspase-3 by Western blotting. A, though a representative Western blot is shown, changes in target protein expression from three independent experiments was semi-quantified by densitometry and presented on the right as fold change over Nx, which was set at a value of 1. The numbers at the bottom in panels F and G denote lane numbers. *P<at least 0.05 vs. Nx; †P<at least 0.05 vs. IH±controls (n=3-6).

IH, TRAF3IP2, NF-κB and HIF-1α activation

We next investigated whether IH activates NF-κB and HIF-1α in SMC. Results in Fig. 2A show that IH induced NF-κB activation as evidenced by increased nuclear phospho-p65 levels, an effect that was inhibited by TRAF3IP2 knockdown or inhibitors of superoxide, H_2O_2, and nitrite production. Similarly, IH-induced HIF-1α activation via a similar signaling pathway, as evidenced by a marked increase in phospho-HIF-1α levels (Fig. 2B). Further, the cell permeable NF-κB inhibitor SN50 or the proteasomal inhibitor MG-132 attenuated IH-induced HIF-1α activation (Fig. 2C). The inhibitory effects of SN50 or MG132 on NF-κB activation was confirmed by Western blotting as shown on the right. Importantly, silencing NF-κBp65 or HIF-1α attenuated IH-induced SMC proliferation (Fig. 2D), without affecting cell viability as evidenced by the low levels of released LDH and caspase-3 activation (Fig. 2E). Together, these results indicate that IH induces NF-κB and HIF-1α activation in part via TRAF3IP2. Moreover, targeting NF-κB inhibits HIF-1α activation (Fig. 2).

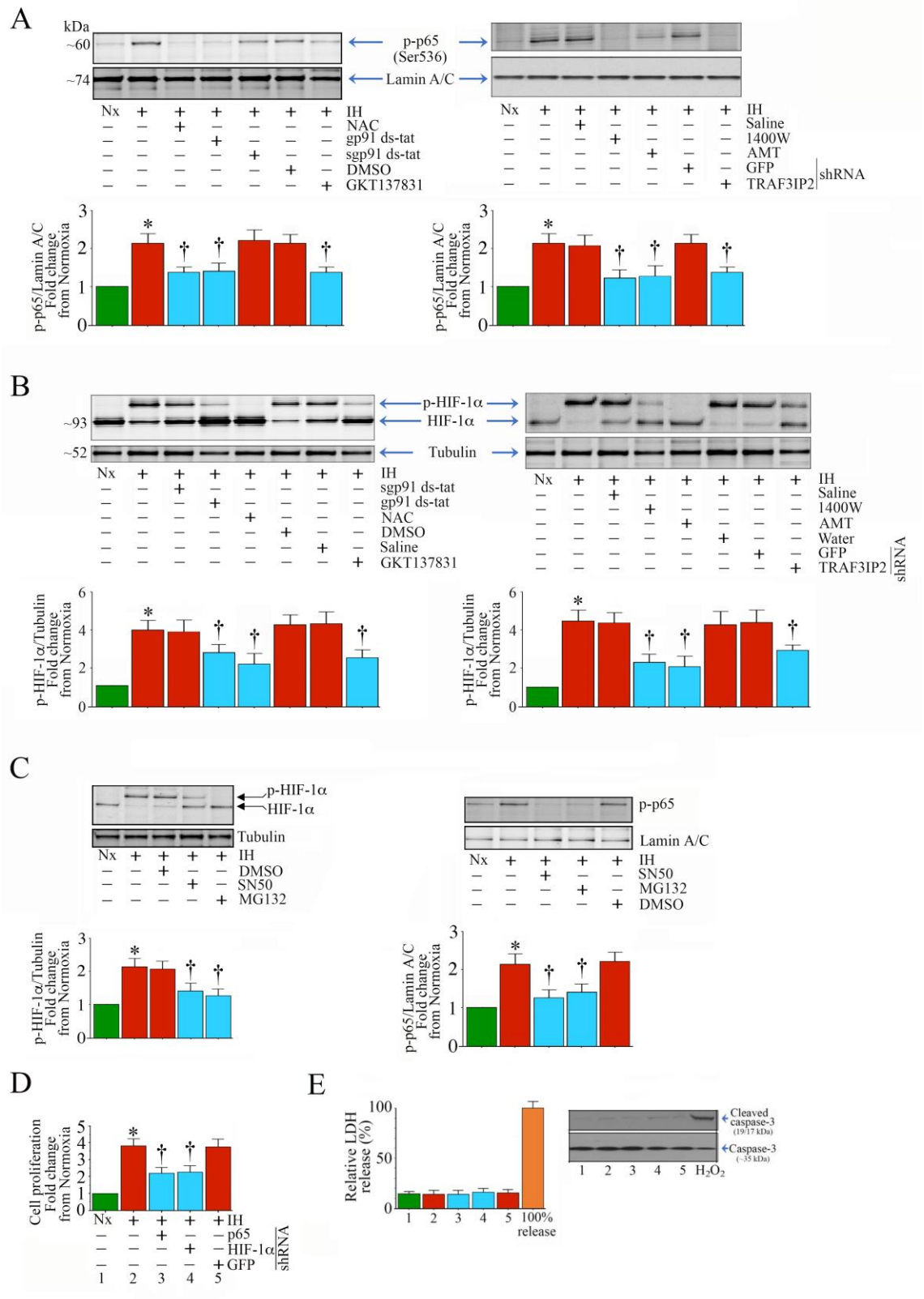

Figure 2. IH induces NF-κB and HIF-1α activation via TRAF3IP2. A, IH activates NF-κB via TRAF3IP2 and nitroxidative stress. Quiescent SMC were treated with NAC, gp91 ds-tat, GKT137831, 1400W or AMT prior to IH (50 cycles), and analyzed for NF-κB activation by Western blotting using equal amounts of nuclear protein extracts (10 μg) and activation-specific p65 antibodies (Ser^{536}). Lamin A/C served as a loading control. In a subset of

experiments, SMC were transduced with Ad.TRAF3IP2 shRNA, made quiescent, and then exposed to IH (right hand panel). B, IH activates HIF-1α via TRAF3IP2 and nitroxidative stress. Quiescent SMC treated as in *A* were analyzed for HIF-1α activation by Western blotting using cleared whole cell lysates (20 μg). Tubulin served as a loading control. C, IH induces HIF-1α activation via NF-κB. Quiescent SMC were treated with the NF-κB inhibitor SN-50 or the proteasomal inhibitor MG-132 prior to IH and analyzed for HIF-1α activation as in *B*. Inhibition of NF-κB activation was confirmed by Western blotting (right hand panel) as in *A*. D, Targeting NF-κB and HIF-1α inhibit IH-induced SMC proliferation without affecting cell viability. SMC transduced with lentiviral shRNA against NF-κBp65 or HIF-1α were made quiescent and exposed to IH. Cell proliferation was analyzed by the CyQUANT™ assay after 48h (D; n=6). Cell viability was analyzed by a colorimetric LDH release assay and activation of caspase-3 by Western blotting (E). A,B,C,E, though a representative Western blot is shown, changes in target protein expression from three independent experiments were semi-quantified by densitometry, and presented at the bottom of respective panels as a fold change over control, which was set at a value of 1. *P<at least 0.05 vs. Nx; †P<at least 0.05 vs. IH±GFP (n=3).

IH, STAT3 phosphorylation, IL-6 induction

IL-6 is a proinflammatory and pro-mitogenic cytokine. We therefore investigated whether IH upregulates IL-6 expression via TRAF3IP2, NF-κB, and HIF-1α. Indeed, IH induced IL-6 mRNA and protein expression in SMC, and this effect was markedly inhibited by silencing NF-κB, HIF-1α, or TRAF3IP2 (Fig. 3A, 3B; knockdown of p65 and HIF-1α was confirmed by Western blotting as shown in Fig. 3C). Further, exposure to IL-6 induced STAT3 phosphorylation in a concentration- and time-dependent manner, with significant effects observed at 30 ng/ml and for 15 min (Fig. 3D). Therefore, in all subsequent experiments, IL-6 was used at 30 ng/ml for 15 min. Since the recombinant IL-6 protein contained trace amounts of endotoxin (<0.10 EU/μg of the protein), and as endotoxin is known to activate STAT3 [26], we next determined the possible contribution of endotoxin to IL-6-mediated STAT3 activation by treating SMC with Polymyxin B sulphate for 2 h prior to IL-6 addition. The results showed no significant inhibition in IL-6 mediated STAT3 activation (Fig. 3D, right hand panel) ruling out the role of trace amounts of endotoxin on STAT3 phosphorylation. Further, the JAK inhibitor Tofacitinibor and the STAT inhibitor HO-3867 attenuated IL-6-induced STAT3 phosphorylation (Fig. 3E). However, silencing IL-6R or gp130 markedly attenuated IL-6-induced STAT3 phosphorylation (Fig. 3E, right hand panel). Knockdown of IL-6R and gp130 was confirmed by Western blotting as shown in Fig. 3F. Together, these results indicate that IH upregulates IL-6 expression, and IL-6 induces STAT3 activation in an IL-6R/gp130/JAK-dependent manner (Fig. 3).

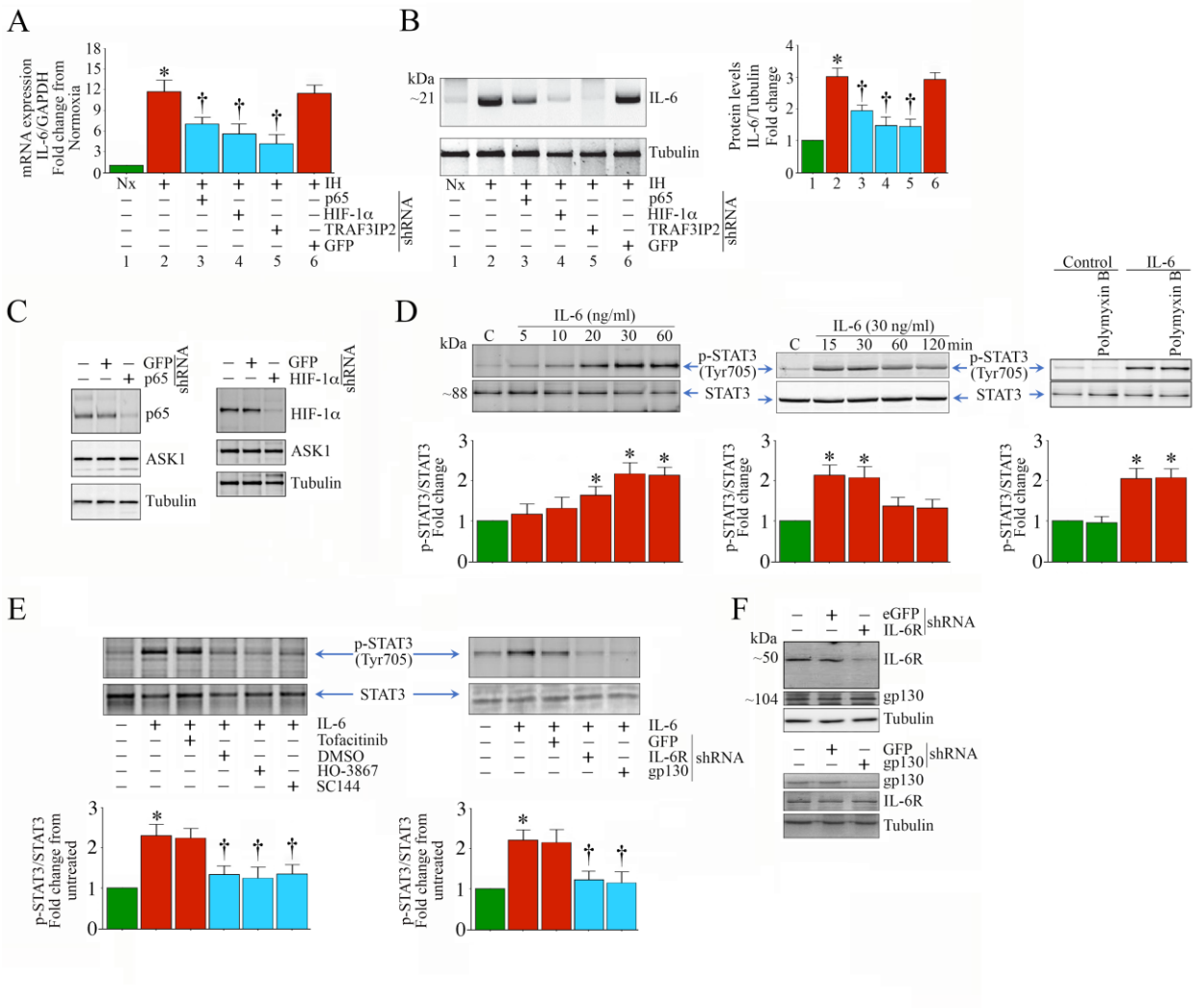

Figure 3. IH induces IL-6 expression via TRAF3IP2. A-C, IH upregulates IL-6 mRNA and protein expression via TRAF3IP2, NF-κB and HIF-1α. SMC were transduced with NF-κBp65, HIF-1α or TRAF3IP2 shRNA using viral vectors, made quiescent, and exposed to 50 cycles of IH. GFP shRNA served as a control. IL-6 mRNA was analyzed by RT-qPCR (A) and protein expression by Western blotting (B). Knockdown of NF-κBp65 and HIF-1α was confirmed by Western blotting (C). ASK1 served as a non-targeting control. Tubulin served as a loading control. D, IL-6 activates STAT3 in a concentration- and time-dependent manner. Quiescent SMC treated with the indicated concentrations of IL-6 (left hand panel) and for up to 120 min at 30 ng/ml (middle panel) were analyzed for total and p-STAT3 levels by Western blotting using cleared whole cell lysates (20 μg). In a subset of experiments, SMC were exposed to Polymyxin B sulphate for 2 h prior to incubation with IL-6 (30 ng/ml for 15 min). STAT3 activation was analyzed by Western blotting (right hand panel). E, F, IL-6 induces STAT3 phosphorylation via IL-6R, gp130 and JAK. Quiescent SMC treated with the gp130 inhibitor SC144, JAK inhibitor HO-3867 and the STAT3 inhibitor Tofacitinib prior to IH were analyzed for STAT3 phosphorylation as in *D*. In a subset of experiments, SMC were transduced with IL-6R or gp130 shRNA, made quiescent, and then exposed to IH (right hand panel). Knockdown of IL-6R and gp130 was confirmed by Western blotting (F). B-F, though a representative Western blot is shown, changes in target protein expression from three independent experiments were semi-quantified by densitometry and presented at the bottom or side of respective panels as a fold change over control, which was set at a value of 1. The numbers at the bottom of panels denote lane numbers. *P<at least 0.05 vs. Nx; †P<at least 0.05 vs. IH (n=3).

IL-6, TRAF3IP2, and SMC proliferation

Exposure to the proinflammarory cytokine IL-6 induced SMC proliferation (Fig. 4A), an effect that was attenuated by IL-6R, gp130, JAK, STAT3 and TRAF3IP2 knockdown or inhibition. However, targeting IL-6R, gp130, JAK or STAT3 did not compromise cell viability, as evidenced by similar and low levels of secreted LDH and cleaved caspase-3 expression (Fig. 4B). Together, these results indicate that TRAF3IP2 mediates IL-6 induced SMC proliferation through TRAF3IP2 and the JAK/STAT3 pathway (Fig. 4).

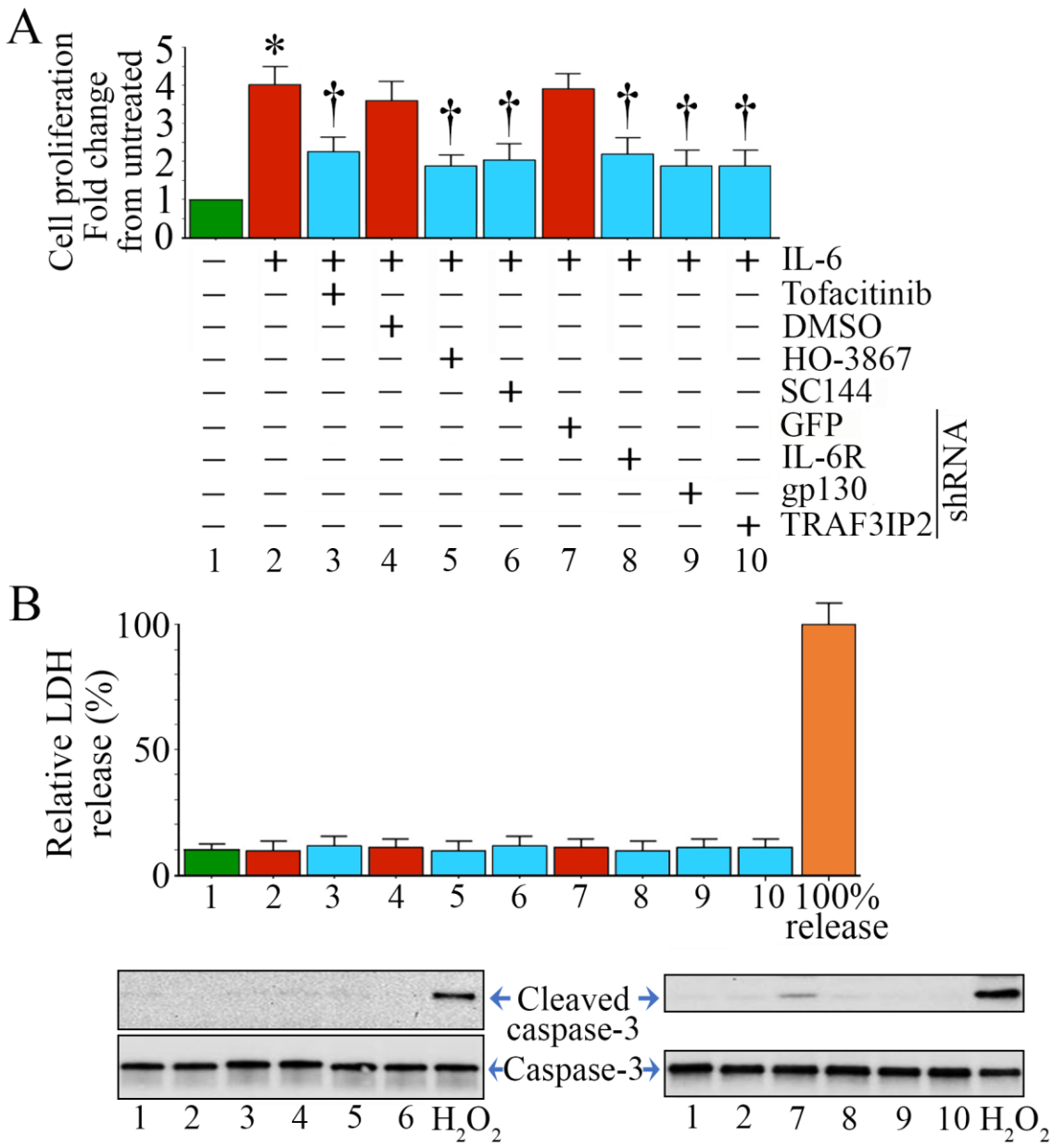

Figure 4. IL-6 stimulates SMC proliferation via IL-6R, gp130, JAK, STAT3 and TRAF3IP2. A, Quiescent SMC treated with the gp130, JAK or STAT3 inhibitor prior to IL-6 addition were analyzed for proliferation at 48 h by the CyQUANT™ assay. In a subset of experiments, SMC were transduced with IL-6 or gp130 or TRAF3IP2 shRNA, made quiescent, and then exposed to IL-6 (n=6). B, Pharmacological inhibitors or shRNA-mediated knockdown of targets described in A did not negatively impact SMC viability. Cell viability was analyzed by LDH release by the LDH-Glo™ Cytotoxicity Assay (n=6) and activation of caspase-3 by Western blotting (bottom panel; n=3). In LDH cytotoxicity assay, treatment of SMC with Triton X-100 served as a positive control and was set to 100% LDH release. H_2O_2 (100 μM) served as a positive control in Western blotting. *P<at least 0.05 vs.saline; †P<at least 0.05 vs. IL-6 (n=3-6).

Empagliflozin and IL-6-induced SMC proliferation

We next investigated whether empagliflozin inhibits IL-6-induced SMC proliferation. At first, we determined whether SMC express SGLT2 and whether IL-6 upregulates its expression. Confirming our previously published results [19], data in Fig. 5A show that

SMC express SGLT2 protein at low levels under basal conditions, and is induced by IL-6. Protein extracts from human kidney and HK-2 cell line served as positive controls, and at a similar protein concentration, the kidney homogenates had higher SGLT-2 expression compared to IL-6 exposed SMC. Further, IL-6 induces SGLT2 expression in a time-dependent manner (Fig. 5B), with peak levels detected after 4 h. Targeting STAT3 by HO-3867 markedly suppressed IL-6-induced SGLT2 expression, and importantly, pretreatment with empagliflozin suppressed IL-6-induced STAT3 phosphorylation, an effect that was partially reversed by SGLT2 knockdown (Fig. 5C). Importantly, empagliflozin inhibited IL-6-mediated SMC proliferation, an effect reversed by SGLT2 knockdown (Fig. 5D), without compromising cell viability as evidenced by similar and low levels of secreted LDH and cleaved caspase-3 expression (Fig. 5D, right hand panels). These results demonstrate that IL-6 induces SGLT2 expression in part via STAT3, and empagliflozin inhibits IL-6's pro-mitogenic effects (Fig. 5).

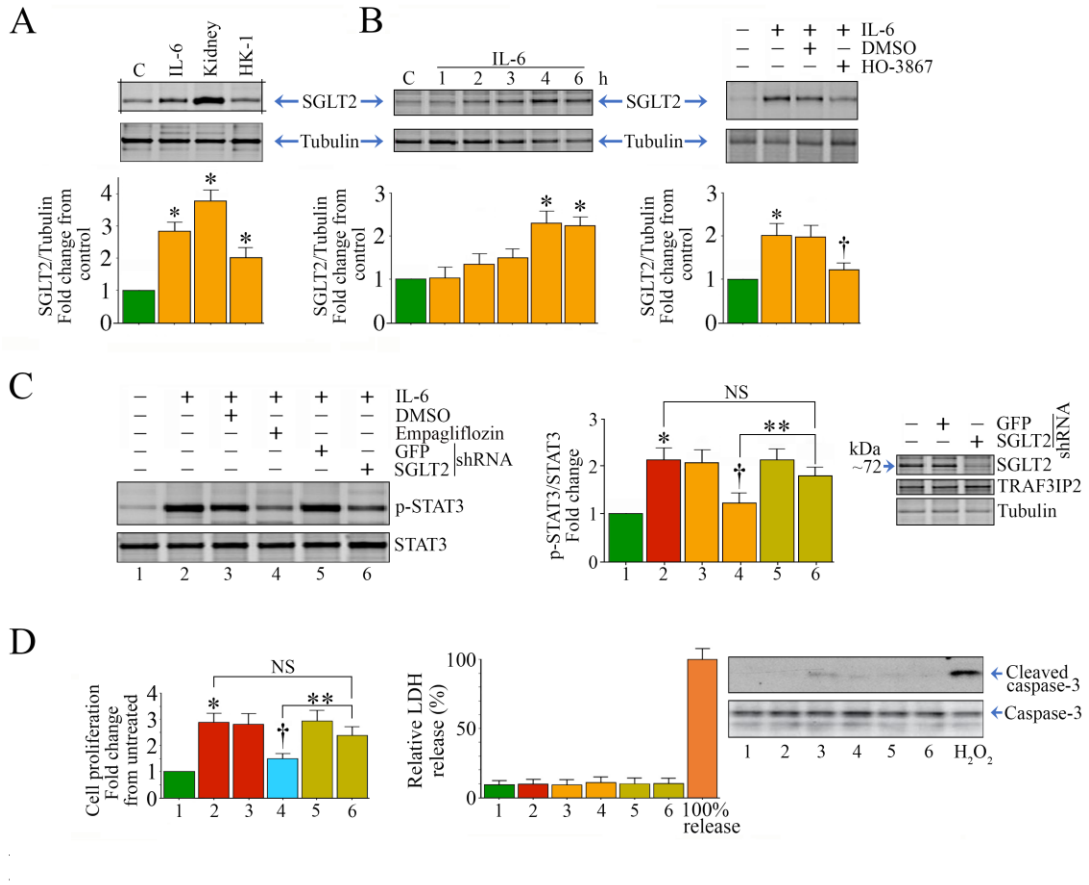

Figure 5. IL-6 induces SGLT2 expression via STAT3. A, IL-6 upregulates SGLT2 expression. Quiescent SMC exposed to IL-6 (30 ng/ml for 4h) were analyzed for SGLT2 expression by Western blotting using equal amounts of whole cell lysates (20 μg). Cell lysates from the human proximal tubule epithelial cells HK-2 and human kidney extracts served as positive controls (20 μg). B, IL-6 upregulates SGLT2 expression via STAT3. Quiescent SMC exposed to IL-6 (30 ng/ml) for up to 6h (left hand panel) were analyzed for SGLT2 expression as in A. In a subset of experiments, quiescent SMC were treated with the STAT3 inhibitor HO-3867 prior to IL-6 addition (30 mg/ml for 4 h). C, Empagliflozin inhibits IL-6-induced STAT3 phosphorylation via SGLT2. Quiescent SMC were exposed to empagliflozin (1 μM for 15 min) prior to IL-6 addition (30 ng/ml for 15 min). Total and phospho-STAT3 levels were analyzed by Western blotting using whole cell lysates. In a subset of experiments, SMC were transduced with SGLT2 shRNA by lentiviral transduction, made quiescent, and then treated with IL-6. Knockdown of SGLT2 was confirmed by Western blotting. TRAF3IP2 served as an off target. D, Empagliflozin or SGLT2 knockdown failed to affect cell viability. Cell viability was analyzed by LDH-Glo™ Cytotoxicity Assay (n=6) and activation of caspase-3 by Western blotting as in Fig. 4B. A-D, though a representative Western blot is shown, changes in target protein expression from three independent experiments were semi-quantified by densitometry, and presented at the bottom or side of respective panels as a fold change over control, which was set at a value of 1. The numbers at the bottom of panels denote lane numbers. *P<at least 0.05 vs.saline; †P<at least 0.05 vs. IL-6; **P<0.05 vs. IL-6+EMPA (n=3-6).

Empagliflozin and IH-induced SMC proliferation

We next investigated whether empagliflozin inhibits IH-induced SMC proliferation. First, we investigated whether IH, similar to IL-6 (Fig. 5), upregulates SGLT2 expression in SMC. Indeed, results in Figure 6A show that, like IL-6, IH upregulated SGLT2 expression in SMC. Further, empagliflozin inhibited IH-induced TRAF3IP2 expression and nitroxidative stress (Fig. 6B). Empagliflozin also inhibited activation of NF-κB, as evidenced by the reduced levels of nuclear phospho-p65 levels (Fig. 6C, upper panel). Similarly, empagliflozin inhibited HIF-1α activation (Fig. 6C, lower panel). Empagliflozin also inhibited IH-induced IL-6 expression and STAT3 phosphorylation (Fig. 6D). Importantly, empagliflozin inhibited IH-induced SMC proliferation (Fig. 6E), without compromising cell viability, as evidenced by similar and low levels of secreted LDH and cleaved caspase-3 expression (Fig. 6E). These results indicate that empagliflozin inhibits IH-induced SMC proliferation by targeting nitroxidative stress, NF-κB and HIF-1α activation, IL-6 expression, and STAT3 phosphorylation (Fig. 6).

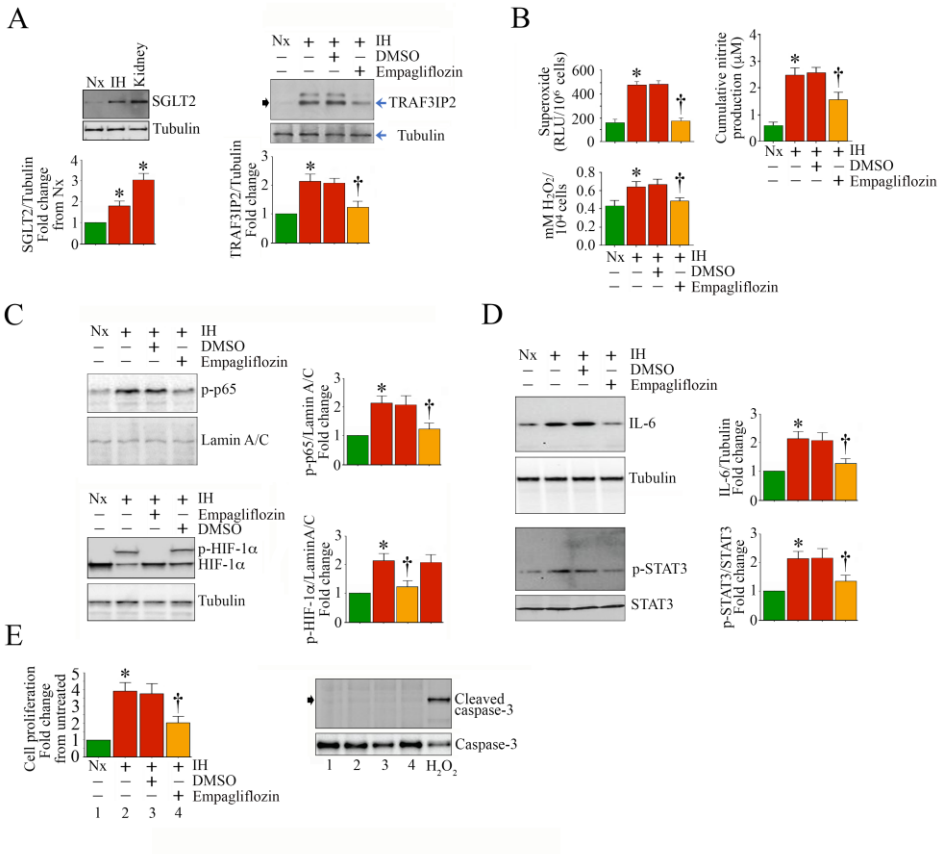

Figure 6. Empagliflozin inhibits IH-induced SMC proliferation. A, IH upregulates SGLT2, and targeting SGLT2 by empagliflozin inhibits IH-induced TRAF3IP2 expression. Quiescent SMC were exposed to IH or Nx for 50 cycles, and analyzed for SGLT2 expression by Western blotting. Human kidney homogenate served as a positive control. In a subset of experiments, quiescent SMC were exposed to empagliflozin (1 μM for 15 min) prior to IH (50 cycles), and then analyzed for TRAF3IP2 expression by Western blotting (n=3). B, Empagliflozin inhibits IH-induced nitrooxidative stress. Quiescent SMC exposed to empagliflozin as in *A* were analyzed for superoxide, H_2O_2 and nitrate+nitrite levels by lucigenin-enhanced chemiluminescence assay, Amplex Red assay and Greiss reaction (n=6). C, Empagliflozin inhibits IH-induced NF-κB and HIF-1α activation. Quiescent SMC exposed to empagliflozin and IH as in A were analyzed for NF-κB activation by Western blotting using equal amounts of nuclear protein extracts and activation-specific anti-p65 antibodies. Total and phospho-HIF-1α levels were analyzed in whole cell lysates by Western blotting. D, empagliflozin inhibits IH-induced IL-6 expression and STAT3 phosphorylation. Quiescent SMC exposed to empagliflozin followed by IH were analyzed for IL-6 expression and phospho-STAT3 levels by Western blotting using cleared whole cell lysates. E, Empagliflozin inhibits IH-induced SMC proliferation without affecting cell viability. Quiescent SMC treated as in *A* were analyzed for proliferation by the CyQUANT™ assay after 48h (n=6). Cell viability was analyzed by LDH-Glo™ Cytotoxicity Assay (n=6) and activation of caspase-3 by Western blotting as in Fig. 4B. *P<at least 0.05 vs. Nx; †P<at least 0.05 vs. IH (n=3-6).

4. Discussion and Conclusions

OSA is a chronic disease and a major public health problem. It is a risk factor for hypertension, stroke, coronary artery disease, and myocardial infarction. Obesity, smoking, diabetes, and aging further increase its incidence. In fact, OSA is associated with and is a causal factor for these comorbid conditions. IH, the underlying cause of OSA and associated complications, contributes to OSA pathology by enhancing oxidative stress,[27] inflammation,[28] and endotheliual dysfunction,[27] among others. Both low grade oxidative stress and chronic inflammation contribute to cardiovascular diseases, including atherosclerotic coronary artery disease. Atherosclerosis is a chronic progressive inflammatory disease with significant morbidity and mortality. Therefore, OSA and associated comorbid conditions are major contreibutors to cardiovascular and all-cause mortality.

TRAF3IP2 is an oxidative stress-responsive proinflammatory adapter molecule and an upstream regulator of various critical transcription factors and stress-acxtivated likases that play a role in atheroscxlerosis development and progrwession.[5,6,11] Therefore, we hypothesized that IH promotes crosstalk between TRAF3IP2 and oxidative stress leading to SMC proliferation, a hallmark of vascular proliferative diseases. We further hypothesized that SGLT2 inhibitors, which exert pleiotropic antioxidant, anti-inflammatory, and anti-mitogenic effects, will blunt IH-indsuced oxidative stress, inflammatory cytokine expression, and SMC proliferation. Supporting our hypothesis, our results show for the first time that IH upregulates TRAF3IP2 expression and TRAF3IP2-deoendent nitropxidative stress, leading to SMC proliferation via NF-κB and HIF-1α activation and IL-6 expression. Exposure to IL-6 by itself induced SMC proliferation in a JAK/STAT3-dependent manner. Further conforming our previous results,[19] SMC expressed SGLT2 at basal conditions, and both IH and IL-6 upregulated its expression. Importantly, we also show for the first time that empagliflozin inhibited IH-induced SMC proliferation by targeting nitroxidative stress, activation of NF-κB and HIF-1α, and STAT3 phosphorylation (Fig. 7). Together, these results suggest the therapeutic potential of empagliflozin in IH and inflammatory vascular proliferative diseases associated with OSA and chronic IH.

Both oxidative stress and inflammation have been shown to contribute to development of IH-mediated cardiovascular diseases (CVD) during OSA in preclinical models. We and others have previously demonstrated that TRAF3IP2 plays a causal role in various animal models of CVD, including ischemia-reperfusion-induced myocardial injury and atherosclerosis [11,29]. Moreover, using genetic models of TRAF3IP2 deletion, we have demonstrated the causal role of TRAF3IP2 in hypertensive heart disease resulting from angiotensin II or aldosterone infusion.[30,31] Here, we report for the first time that IH, a consequence of OSA, upregulates TRAF3IP2 expression in SMC. Moreover, IH stimulated superoxide, H_2O_2 and nitric oxide production in part via TRAF3IP in SMC. Interestingly, the antioxidant NAC and inhibitors of Nox2, Nox4, and iNOS suppressed IH-induced TRAF3IP2 expression in SMC, suggesting that TRAF3IP2 is both upstream and downstream of oxidative stress. In fact, increased oxidative stress has been reported in subjects with OSA, as evidenced by increased production of Nox-dependent superoxide and lipid peroxidation products, and their suppression following CPAP [32,33]. Interestingly, in OSA subjects with comorbid conditions, the levels of superoxide were found to be much more elevated. Therefore, targeting TRAF3IP2 has the therapeutic potential in OSA-associated complications by suppressing oxidative stress (Fig. 7).

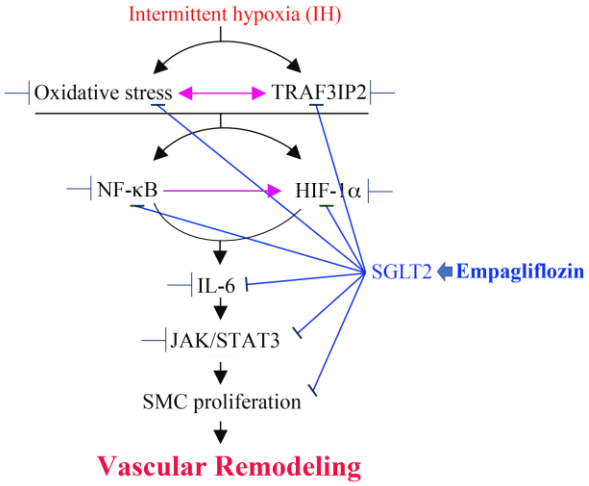

Figure 7. A working model describing the molecular mechanisms underlying IH and IL-6-induced SMC proliferation, and those targeted by empagliflozin. IH induces primary human aortic SMC proliferation via the crosstalk between TRAF3IP2 and nitrooxidative stress, NF-κB and HIF-1α activation, and induction of IL-6 and

activation of its downstream signaling. Moreover, IL-6 induced SGLT2 expression in part via STAT3 and targeting SGLT2 by empagliflozin attenuated STAT3 phosphorylation and SMC proliferation without affecting cell viability. IH also upregulated SGLT2 expression and targeting SGLT2 by empagliflozin attenuated IH-induced TRAF3IP2 expression, NF-κB and HIF-1α activation, IL-6 expression, STAT3 phosphorylation and SMC proliferation. Together, these mechanistic *in vitro* results suggest the therapeutic potential of empagliflozin in vascular proliferative diseases.

TRAF3IP2 is an upstream regulator of critical oxidative stress-responsive nuclear transcription factors NF-κB, AP-1, c/EBP, and p38 MAPK that play a role in the pathogenesis of OSA in patients and preclinical models [34,35]. Here we demonstrate that targeting TRAF3IP2 significantly reduced IH-induced NF-κB activation. Moreover, targeting TRAF3IP2 reduced HIF-1α activation. Though we and others have reported the mechanisms underlying TRAF3IP2-mediated NF-κB activation [5,6,30], we did not investigate the molecular mechanisms underlying TRAF3IP2-dependent HIF-1α activation. It is plausible that stress-activated kinases, such as p38MAPK and JNK [36], which are downstream of TRAF3IP2, might have contributed to IH-induced TRAF3IP2-dependent HIF-1α phosphorylation/activation. Our results also show that targeting NF-κB suppressed HIF-1α activation as reported previously [37]. Though we did not investigate whether targeting HIF-1α regulates NF-κB activation, a crosstalk has been reported previously between NF-κB and HIF-1α in regulating inflammation. For example, HIF-1α has been shown to regulate epithelial inflammation by cell autonomous NF-κB activation and paracrine stromal remodeling [38]. On the other hand, targeting HIF-1α has been shown to restrict NF-κB-dependent gene activation, potentially to blunt excessive and damaging pro-inflammatory response [39]. These reports suggest that regulation of NF-κB by HIF-1α appears to be cell type- and context-dependent, and our future studies will determine whether targeting HIF-1α blunts IH-induced NF-κB activation in SMC. .

Both NF-κB and HIF-1α transcriptionally upregulate various inflammatory cytokines, including IL-6. Here we show that targeting NF-κB and HIF-1α each attenuated IH-induced IL-6 expression. Similarly, targeting TRAF3IP2 suppressed IH-induced IL-6 expression. We also show that IL-6 treatment stimulated SMC proliferation in part via STAT3 activation. Together, these results suggest a positive correlation between IH and IL-6 expression. In fact, using meta-analysis and meta-regression aimed at comparing systemic levels of IL-6 between young and adult subjects, it was recently reported that IL-6 levels are significantly elevated in subjects with OSA compared to controls [40]. Moreover, a positive correlation was reported between elevated systemic IL-6 levels and CVD, a consequence of OSA [41].

For the first time, our results show that both IH and IL-6 upregulate SGLT2 expression in SMC, and exposure to the SGLT2 inhibitor empagliflozin attenuated IH- and IL-6-induced SMC proliferation. Though we have not investigated the mechanisms underlying IH and IL-6 induced SGLT2 induction, it was previously reported that the *SGLT2* proximal promoter region contains multiple NF-κB binding sites, and deletion of the promoter region between -1700 to -2000 region significantly reduces SGLT2 promoter-dependent reporter gene activity in HK-2 cells [42], demonstrating NF-κB regulation of *SGLT2* transcription. Our results also show that targeting STAT3 attenuated IL-6-induced SGLT2 expression, suggesting a potential role for STAT3 in *SGLT2* transcription, which will be investigated in our future studies. We also demonstrate that empagliflozin inhibited IL-6-induced STAT3 phosphorylation, and this effect was reversed by SGLT2 knockdown, demonstrating that empagliflozin signaled mainly via SGLT2. Similarly, pretreatment with empagliflozin inhibited IH-induced TRAF3IP2 expression, nitroxidative stress, NF-κB and HIF-1α activation, IL-6 expression, and STAT3 phosphorylation.

These *in vitro* mechanistic studies suggest the therapeutic potential of SGLT2 inhibitors in OSA with associated comorbid conditions. In fact, SGLT2 inhibitors are widely used to reduce blood glucose levels in subjects with diabetes, a comorbid condition in OSA. Due to their pleotropic effects, SGLT2 inhibitors are also approved as an adjunct therapy in heart failure, another comorbid condition associated with OSA. Because OSA is frequently associated with obesity, diabetes, hypertension, kidney, and cardiovascular complications, we hypothesize that SGLT2 inhibitors have the therapeutic potential in OSA with these comorbidities. In fact, SGLT2 inhibitors have been suggested to be beneficial in patients with OSA along with current surgical interventions and CPAP devices. In studies involving empagliflozin and other gliflozins, it was reported that these agents reduced both cardiovascular and all-cause mortality by reducing blood glucose levels and improving HbA1c, and systolic and diastolic dysfunction.[43] While these studies showed improvement in metabolic, cardiovascular and renal outcomes in OSA subjects with comorbidities, it is however not known whether gliflozins have a positive impact in OSA subjects without comorbidities. Though our cell culture studies are considered a limitation compared to studies in preclinical models and clinical trials, these mechanistic studies demonstrate that the SGLT2 inhibitor empagliflozin inhibits TRAF3IP2-dependent pro-oxidant, proinflammatory, and pro-mitogenic effects in SMC exposed to IH (Fig. 7), and suggest the therapeutic potential of empagliflozin or

other SGLT2 inhibitors in OSA and diseases associated with chronic IH.

Conclusions:

Obstructive sleep apnea (OSA) is a chronic sleep-associated breathing disorder. OSA often results in intermittent hypoxia (IH) consequent to cyclical apneas and hypopneas, resulting in persistent oxidative stress and inflammation in various organs, including the heart and blood vessels. Our *in vitro* mechanistic studies demonstrate that treatment with empagliglozin, an SGLT2 inhibitor, inhibited IH-induced nitroxidative stress, TRAF3IP2 induction, NF-κB and HIF-1α activation, and IL-6 upregulation. Treatment with empagliflozin also inhibited IL-6-induced STAT3 phosphorylation and SMC proliferation. These results suggest the therapeutic potential of SGLT2 inhibitors in OSA and associated complications.

Conflicts of interest

No potential conflicts of interest.

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Author contributions

YH, RSR and BC conceived the experiments; RD, YH, NAD, JJR, LAM-L, RSR and BC contributed to data collection and formal analysis. RD, YH, LAM-L, RSR and BC wrote the manuscript, and all authors reviewed/edited the manuscript, and approved the final version.

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