NF-κB pathway is involved in CRP-induced effects on pulmonary arterial endothelial cells in chronic thromboembolic pulmonary hypertension

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Wynants M, Vengethasamy L, Ronisz A, Meyns B, Delcroix M, Quarck R. NF-κB pathway is involved in CRP-induced effects on pulmonary arterial endothelial cells in chronic thromboembolic pulmonary hypertension. Am J Physiol Lung Cell Mol Physiol 305: L934–L942, 2013. First published October 4, 2013; doi:10.1152/ajplung.00034.2013.—Chronic thromboembolic pulmonary hypertension (CTEPH) is characterized by thrombofibrotic obstruction of proximal pulmonary arteries. The cellular and molecular mechanisms underlying the pathogenesis remain incompletely understood, although we recently evidenced the potential involvement of the inflammatory marker C-reactive protein (CRP). We aimed to investigate the intracellular mechanisms induced by CRP in proximal pulmonary arterial endothelial cells (PAEC). PAEC were isolated from vascular material obtained during pulmonary endarterectomy. RNA was extracted from CRP-stimulated PAEC, and first-stand cDNA was generated. A RT2 profiler PCR Array was used to evaluate the expression of 84 key genes related to NF-κB-mediated signal transduction. CRP-induced NF-κB activation was studied. The effects of pyrrolidine-dithio-carbamate ammonium (PDTC), an inhibitor of the NF-κB pathway, were investigated on NF-κB-mediated adhesion of monocytes to PAEC, adhesion molecule expression, endothelin-1 (ET-1), interleukin-6 (IL-6), and von Willebrand factor (vWF) secretion. Compared with nonstimulated PAEC, CRP receptor 2B was downregulated by 25%, inhibitor of NF-κB kinase subunit epsilon (IKBKE) by 30%, and toll-like receptor 4 and -6 by 18 and 39%, respectively, in CRP-stimulated PAEC. The transcription factor FOS was threefold upregulated. CRP induced RelA/NF-κBp65 phosphorylation. PDTC dose dependently inhibited the adhesion of monocytes to CRP-stimulated PAEC. PDTC also inhibited the CRP-induced expression of ICAM-1 at the surface of PAEC. PDTC impaired the secretion of ET-1 by 18% and tended to inhibit the secretion of IL-6 by 46% of CRP-stimulated PAEC. PDTC did not inhibit the CRP-induced secretion of vWF. These results suggest an involvement of the NF-κB pathway in mediating different effects of CRP on proximal CTEPH-PAEC.

C-reactive protein; endothelial cells; NF-κB; chronic thromboembolic pulmonary hypertension

CHRONIC THROMBOEMBOLIC PULMONARY hypertension (CTEPH) is a severe cause of pulmonary hypertension characterized by an obliteration of proximal pulmonary arteries by intraluminal thrombi and fibrous stenosis. Pulmonary embolism, either as single or recurrent episodes, is thought to be the initiating event followed by progressive pulmonary vascular remodeling. Vascular disobliteration by pulmonary endarterectomy (PEA) is the preferred treatment for CTEPH patients (38). Knowledge of the physiopathology of CTEPH remains incomplete. Proximal lesions share similarities with atherosclerotic plaques, including media thickening and neointima formation (1). Risk factors for CTEPH include size of the initial thrombus, elevated factor VIII, circulating phospholipid antibodies, and fibrinogen intrinsic abnormalities, as well as medical conditions such as splenectomy, cancer, ventriculotrial shunt, chronic inflammatory disease, and hypothyroidism (19). More recently concepts of inflammatory thrombosis and deficient angiogenesis have been introduced (21). For instance, staphylococcal infection delayed thrombus resolution in mice (5), suggesting that bacterial infection could impair thrombus resolution in CTEPH. In addition, enhanced proliferative features have been observed in pulmonary vascular cells harvested from PEA specimens (32, 40) and endothelial and mesenchymal progenitor cells have been identified in pulmonary vascular tissue harvested from CTEPH patients during PEA (10, 54). C-reactive protein (CRP), an acute phase protein, is a well-known marker of inflammation and tissue damage. Elevated circulating CRP levels are an independent risk factor for cardiovascular disease, including myocardial infarction, stroke, and atherosclerosis (20). Besides being a biomarker, CRP also plays an active role in atherogenesis (9, 44). Indeed, CRP locally induces the production of vasoconstrictive, thrombotic, proliferative, and inflammatory molecules (8). Several reports suggest that CRP effects on endothelial (EC) and smooth muscle cells (SMC) within the vascular wall could be partially mediated via the nuclear factor-κB (NF-κB) pathway (7, 13, 18, 24).

The NF-κB superfamily is a family of transcription factors, involved in a broad range of biological processes, including the immune response, cell survival, stress response, and cell maturation. NF-κB transcription factor family consists of five genes, namely NFKB1, NFKB2, RELA, REL, and RELB, which encode RelA (p65), RelB, c-Rel, p50, and p52 proteins, respectively. Intracellular forms of NF-κB include homo- or heterodimers comprised by its subunit association. Inactivated NF-κB dimers are associated with inhibitors, namely IkB, inhibiting their nuclear translocation and subsequent DNA binding. NF-κB activation involves phosphorylation-dependent degradation of IkB proteins via a proteasome-regulated pathway including IkB kinases (IKK) resulting in NF-κB nuclear targeting (17).

We have recently shown that circulating levels of CRP are elevated in CTEPH patients and decrease significantly after PEA (39). Moreover, we have provided evidence that CRP is involved in promoting vascular remodeling and endothelial dysfunction in proximal vascular cells harvested from PEA speci-
C-reactive protein (CRP) and hemodynamic parameters were measured at the time of diagnosis, 2.9 ± 2.4 mo before pulmonary endarterectomy. 6MWD, 6 min walking distance; BMI, body mass index; mPAP, mean pulmonary arterial pressure (measured by right heart catheterization); PVR, pulmonary vascular resistance; CI, cardiac index; F, female; M, male.

mens of CTEPH patients. In this regard, we have shown that CRP enhances proximal pulmonary arterial SMC (PASMC) mitogenic activity, inflammatory cell adhesion to proximal pulmonary arterial EC (PAEC) via intercellular adhesion molecule-1 (ICAM-1) and PAEC secretion of endothelin-1 (ET-1), and von Willebrand factor (vWF) in CTEPH (51). We also investigated the effects of CRP on proximal PAEC isolated from patients with nonthromboembolic pulmonary hypertension as well as from lung transplantation donors and observed that CRP had no effect on adhesion capacity, ICAM expression, and ET-1 secretion.

Consequently, we aimed to investigate the intracellular mechanisms induced by CRP in previously characterized (40) primary cultured PAEC of CTEPH patients exclusively. We evaluated the effects of 1) CRP on NF-κB activity and on the expression of genes related to NF-κB signaling pathway in PAEC, and 2) NF-κB pathway inhibition on CRP-induced adhesion of PAEC to monocytes, adhesion molecule expression, ET-1, cytokine, and vWF secretion.

**MATERIALS AND METHODS**

The study protocol was approved by the Institutional Ethics Committee of the University Hospital Leuven, and participants gave written informed consent.

**Materials.** TRIZol, SuperScript III First-Strand Synthesis System and Alexa Fluor 594 goat anti-rabbit antibodies were purchased from Life Technologies (Merelbeke, Belgium). Highly purified human recombinant CRP, tumor necrosis factor-α (TNF-α), phospho-RelA/NF-κB p65 (S536) Cell-Based ELISA, and Proteome Profiler Human Cytokine Array kit were purchased from R&D Systems (Abington, UK). [3H]thymidine (74 GBq/mmol) was from Perkin Elmer (Zaventem, Belgium). Anti-human CD31 antibody coupled to allophycocyanin fluorochrome (CD31-APC, human was provided from Miltenyi Biotec, Utrecht, The Netherlands). The RT2 Profiler PCR array, RT2 SYBR Green/ROX qPCR Master Mix RNeasy Mini Kit, and RNase-
Toll-like receptor 4

5-HTR2B

NF-κB pathway was investigated using a RT2 Profiler PCR Array. Total RNA was extracted and converted to cDNA. The expression of 84 primer pairs isolated from CTEPH patients (n protein (CRP)-stimulated PAEC. Pulmonary arterial endothelial cell (EC) characterization. Primary PAEC were characterized as previously described (40). Briefly, PAEC phenotype was characterized by labeling cells with diI-Ac-LDL and by immunofluorescence using monoclonal antibodies against human CD31 and human vWF. In addition, PAEC were characterized by fluorescent-activated cell sorting using antibodies against human CD31, coupled to allophycocyanin fluorochrome (Miltenyi Biotec). PAEC derived from CTEPH patients expressed CD31 at their surface contained vWF in Weibel-Palade bodies and are able to take up acetylated LDL (Fig. 1, A–C). Moreover, among the 93.9 ± 3.7% viable PAEC derived from CTEPH patients, 94.1 ± 2.3% expressed CD31 at their surface (Fig. 1E).

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RNA isolation and reverse transcription. Total RNA was extracted from primary PAEC by a single-step method (TRIzol) based on the guanidium isothiocyanate acid phenol method, followed by on column-based purification using RNeasy mini kit. Genomic DNA was removed by enzymatic digestion with DNaseI. First-strand cDNA was generated from total RNA by reverse transcription using SuperScript III system.

PCR array. First-strand CDNA was mixed with RT² SYBR Green/ROX qPCR Master Mix, and the mixture was added into a 96-well RT² RNA PCR Array including 84 primer pairs of NF-κB signaling pathway key genes. Relative expression was calculated using the comparative ΔCt method (43). Results of CRP-stimulated cells were compared with those of nonstimulated cells. The CRP dose of 10 μg/ml used in in vitro cell assays has been determined according to the circulating levels of CRP from CTEPH patients (9.0 mg/l), as previously described (39).

NF-κB activity. Confluent PAEC were starved for 24 h in M199 medium supplemented with 0.2% FBS. EC were stimulated with CRP (10 μg/ml) for 3 h in fresh medium and then fixed in PBS containing paraformaldehyde 4% for 20 min. Cells were incubated in a blocking buffer for 1 h at room temperature and with a mixture of rabbit anti-phospho-RelA/NF-κBp65 and mouse anti-RelA/NF-κBp65 primary antibodies for 16 h at 4°C. After washing steps, cells were incubated with a mixture of horseradish peroxidase-conjugated donkey anti-rabbit IgG and alkaline phosphatase-conjugated donkey anti-mouse IgG for 2 h at room temperature. Fluorogenic detection was performed by successively incubating cells with fluorogenic substrates for horseradish peroxidase and for alkaline phosphatase for 20–60 min each, using a fluorescent plate reader with excitation at

Fig. 2. Differentially expressed genes related to NF-κB pathway in C-reactive protein (CRP)-stimulated PAEC. Pulmonary arterial endothelial cell (EC) isolated from CTEPH patients (n = 9) were stimulated for 3 h with CRP. RNA was extracted and converted to CDNA. The expression of 84 primer pairs related to NF-κB pathway was investigated using a RT² Profiler PCR Array. Only significantly differentially expressed genes are shown. Results are expressed as relative expression of gene of interest to that of housekeeping gene.

A: FOS; B: serotonin receptor type 2B (5-HTR2B); C: NF-κB; D: Toll-like receptor (TLR)-4; E: TLR-6. Paired t-test: *P < 0.05, **P < 0.005 vs. control in absence of CRP.
Fig. 3. Effect of CRP on NF-κB activation in PAEC. PAEC were incubated in the absence or in the presence of 10 μg/ml CRP for 3 h and RelA/NF-κBp65 phosphorylation was measured. Results are expressed as the fold increase in phosphorylated RelA/NF-κBp65 over total RelA/NF-κBp65 vs. control without CRP. Experiment was performed on PAEC isolated from left and right pulmonary vascular material of CTEPH patient 92 and from left pulmonary vascular material of CTEPH patient 94. *P = 0.02 vs. control.

540 nm and emission at 600 nm to measure phosphorylated RelA/NF-κBp65 and with excitation at 360 nm and emission at 450 nm to measure total RelA/NF-κBp65. Results are expressed as the ratio of phosphorylated RelA/NF-κBp65 over total RelA/NF-κBp65.

EC adhesion. Confluent PAEC were starved for 24 h in M199 medium supplemented with 0.2% FBS. Increasing concentrations of PDTC, an inhibitor of the NF-κB pathway, were added for 1 h. EC were stimulated with CRP (10 μg/ml) for 3 h in fresh medium. Radiolabeled human monocytic U937 cell adhesion to EC monolayer was quantified as previously described (51).

Cytotoxicity. The cytotoxic response was evaluated with an MTT measurement (35). Briefly, EC were incubated with 10 mM PDTC for 1 h, washed with PBS, and incubated for 3 h with MTT dissolved in HBSS (0.5 mg/ml). The formazan product was then dissolved in DMSO, and absorbance was recorded at a 550-nm wavelength and a reference wavelength of 655 nm. Results are presented as a percentage of values from the nonexposed cells.

Adhesion molecule expression. Cells seeded onto gelatin-coated chamber slides were incubated with 10 mM PDTC for 1 h, washed with PBS, and incubated for 3 h with MTT dissolved in HBSS (0.5 mg/ml). The formazan product was then dissolved in DMSO, and absorbance was recorded at a 550-nm wavelength and a reference wavelength of 655 nm. Results are presented as a percentage of values from the nonexposed cells.

Measurement of ET-1, cytokine, and vWF secretion. PAEC were incubated with 10 mM of PDTC prior 3-h stimulation with 10 μg/ml CRP. After a wash with PBS, cells were incubated in fresh 0.2% FBS medium for 18 h. Conditioned medium was collected, and levels of ET-1, vWF, and different cytokines were measured. ET-1 levels were measured as previously described (51). Cytokines were measured using a Proteome Profiler assay according to the manufacturer’s instructions. Cytokine arrays allow the detection of 36 different cytokine, chemokine and acute phase protein relative levels. vWF antigen concentrates were determined by an ELISA with rabbit polyclonal anti-human vWF as described elsewhere (45).

Statistical analysis. Statistical analyses were performed using SAS Enterprise Guide 4.1 (SAS, Cary, NC) and GraphPad Prism 4.01 (GraphPad Software, La Jolla, CA). In vitro assays were performed on PAEC isolated from different patients, as mentioned by the “n” number in the figure legends. Regarding RelA/NF-κBp65 phosphorylation, monocyte adhesion to PAEC, and ET-1, interleukin-6 (IL-6), and vWF secretion, a large variability was observed within basal levels in starved PAEC; consequently, to standardize variability between patients, results were expressed as fold increase vs. the control in absence of CRP. Differences between three groups or two groups were analyzed using ANOVA test followed by post hoc tests (Tukey) or paired Student t-test, respectively. A value of P < 0.05 was considered statistically significant. All P values were for two-sided tests.

RESULTS

Expression of NF-κB pathway-related genes in CRP-stimulated PAEC. We first investigated mRNA expression levels of various NF-κB pathway-related genes. As shown in Fig. 2, the serotonin receptor type 2B (5-HTR2B) was significantly downregulated by 25% in CRP-stimulated PAEC vs. nonstimulated PAEC. Inhibitor of NF-κB kinase subunit epsilon (IKBKE), and toll-like receptor (TLR)-4 and -6 were also significantly downregulated by 30%, 18 and 39%, respectively. The early transcription factor FOS (FBJ osteosarcoma oncogene) was significantly upregulated by threefold.

CRP-induced NF-κB activation. In a second approach, we investigated the direct effects of CRP on NF-κB activation in PAEC in vitro. CRP significantly induced RelA/NF-κBp65 phosphorylation by 25% in PAEC, indicating that CRP is able to activate NF-κB pathway in PAEC (Fig. 3).

Effects of NF-κB signaling pathway inhibition on CRP-induced effects. To confirm NF-κB-mediated effects of CRP on PAEC, we investigated the effects of PDTC, an inhibitor of the NF-κB pathway, on CRP-induced PAEC adhesion capacity and ET-1 and vWF secretion by PAEC. PDTC inhibits the degradation of IkB thereby preventing nuclear targeting of NF-κB. Cytotoxicity of a broad range of different concentrations of PDTC was first tested. A dose response was performed to detect any potential toxicity of PDTC. Cell viability was >90% at all used concentrations (1, 0.5, 0.1, and 0.05 mM) of PDTC (Fig. 4A). Moreover, we did not observe any change in the morphology of PAEC after 1 h of incubation with PDTC at all used concentrations compared with control PAEC without PDTC (Fig. 4, B–F).

Fig. 4. Cytotoxicity of pyrrolidine-dithio-carbamate ammonium (PDTC). PAEC were incubated with increasing concentrations of PDTC for 1 h. A: cell viability was measured using an MTT test. Results are expressed as % viable cells vs. control in absence of PDTC. Micrographs of PAEC incubated with no PDTC (B), 0.01 mM (C), 0.05 mM (D), 0.1 mM (E), and 1 mM (F) PDTC for 1 h.
PDTC dose dependently inhibited the adhesion of monocytes to CRP-stimulated PAEC, with a maximum decrease of 35% at 1 mM of PDTC (P = 0.02; Fig. 5A). The experiment was repeated with PAEC isolated from seven CTEPH patients showing that PDTC significantly inhibited CRP-induced adhesion of PAEC to monocytes by 33% (P = 0.0009; Fig. 5B).

PDTC inhibitory effect on monocyte adhesion to PAEC was complete since CRP-induced adhesion in the presence of PDTC was similar to that in the absence of CRP. PDTC also inhibited CRP-induced ICAM-1 expression at PAEC surface (Fig. 6).

PDTC significantly inhibited the secretion of ET-1 by 18% (P = 0.008) to reach the value of non-stimulated PAEC (Fig. 7A). Among the various cytokines tested, PDTC reduced CRP-induced IL-6 secretion by 46%, without reaching statistical significance (P = 0.05). The IL-6 level in PDTC-treated PAEC was even lower than the level in nonstimulated PAEC (Fig. 7B). Finally, PDTC did not have any effect on CRP-stimulated vWF secretion by PAEC (Fig. 7C).

DISCUSSION

The present study showed a potential involvement of NF-κB signaling pathway in mediating effects of CRP on PAEC of CTEPH patients. We found that CRP induced NF-κB activation in PAEC from CTEPH patients. In addition, we observed that 5-HTR2B, TLR-4, TLR-6, and IKBKE were significantly downregulated in CRP-stimulated PAEC, whereas FOS transcription factor was significantly upregulated. Furthermore, PDTC, an NF-κB inhibitor, reduced CRP-induced adhesion of monocytes to PAEC, via the adhesion molecule ICAM, and CRP-induced ET-1 and IL-6 secretion, whereas it did not impair CRP-induced vWF secretion by PAEC.

Despite well-described clinical characteristics of CTEPH, the pathological mechanisms underlying the disease development remain poorly understood. Pulmonary vascular remodeling and inflammation may significantly contribute to the fibrothrombotic obstruction of proximal pulmonary arteries in CTEPH (42). Previous analysis of PEA material from >200 specimens has revealed cellular proliferation within proximal recanalized pulmonary arterial lumens and interluminal stroma (2), suggesting that unknown stimuli towards cellular proliferation and inflammatory infiltration might be involved in the pathogenesis of CTEPH. We previously suggested that CRP could contribute to pulmonary arterial cell dysfunction and vascular remodeling in CTEPH by enhancing PASMC mitogenic activity and monocyte adhesion to PAEC, ET-1, and vWF secretion by PAEC (51). CRP-induced effects have been demonstrated to be mediated through the NF-κB pathway in aortic and umbilical vein EC (7, 18, 24). Moreover, contribution of the NF-κB signaling pathway to enhanced inflammatory response has been evidenced in patients with unstable angina and elevated circulating CRP levels (28). Cytokine-mediated EC activation implies short-term effects including adhesion molecule exposition at the EC surface and secretion of thrombogenic activity and monocyte adhesion to PAEC, ET-1, and vWF secretion by PAEC.
for 3 h (CRP) or pretreated by 1 mM PDTC for 1 h before CRP stimulation. P

PAEC at 24 h could be observed (51). since any long-term effects of CRP on proliferation of CTEPH-

The effects of CRP on NF-κB pathway-related gene expression, growth factors. Similarly, we have investigated short-term effects of CRP on NF-κB pathway-related gene expression, corresponding to early steps of CRP-induced EC activation, since any long-term effects of CRP on proliferation of CTEPH-PAEC at 24 h could be observed (51).

Expression of genes related to NF-κB pathway in CRP-stimulated PAEC. In this study, we observed CRP-induced upregulation of FOS, a proto-oncogene belonging to the immediate early gene family of transcription factors. FOS family members upregulate the transcription of various genes involved in proliferation, differentiation, and defense against invasion and cell damage. Interestingly, FOS transcript levels are more significantly associated with severe atherosclerosis than plasma CRP levels (37). Moreover, statin treatment can result in both circulating CRP and mononuclear cell FOS mRNA downregulation (16). Simvastatin may also inhibit NF-κB-mediated effects of CRP on monocyte adhesion to EC and adhesion molecule expression by EC (24, 26). Although statin treatment did not show any significant effects on primary outcome in CTEPH patients (55), it significantly decreases the risk of recurrent pulmonary embolism (3), a risk factor for CTEPH. Additionally, a FOS binding site has been found within the ET-1 gene promoter (23). Finally, CRP promoted NF-κB and AP-1 (c-FOS + c-JUN) transcriptional activity and induced c-FOS and IL-6 mRNA expression in cultured rat aortic SMC (12). This suggests that CRP-induced ET-1 production could be regulated via FOS and/or NF-κB signaling pathways. In the present study, we observed that CRP-induced ET-1 secretion by PAEC was inhibited by PDTC, an inhibitor of NF-κB, suggesting that ET-1 production by PAEC could be driven by NF-κB. However, whether NF-κB is involved in ET-1 synthesis and/or secretion still needs to be determined.

We also found that CRP downregulated the expression of 5-HTR2B in PAEC. 5-HTR2B is expressed in human PAEC and stimulates calcium release (47). 5-HTR2B elicits a reversible endothelium-dependent relaxation of pulmonary arteries in pigs, associated with an increase in cyclic GMP (11), through coupling to NO signaling pathway (30). Loss of function of 5-HTR2B has been incriminated in fenfluramine-associated PAH (4). Considering that transcriptional regulation of serotonin receptor 2B remains controversial and has not been completely unraveled (31), one may hypothesize that down-regulation of 5-HTR2B may result in decreased vasodilation and could contribute to increased pulmonary vascular resistance in CTEPH.

TLRs are receptors involved in innate immune response, expressed by inflammatory and vascular cells (6). TLRs also play a crucial role in inflammation-associated diseases (48), including diabetes mellitus (56) or atherosclerosis (33). Most studies have shown a proatherogenic role for TLRs (34). TLR-4 activation triggers a robust inflammatory response in microvascular EC (29) and in human progenitor EC in vitro (52). However, the precise role of TLRs in the physiopathology of CTEPH should be further investigated.

IKBKE, also named IKKe, phosphorylates inhibitors of NF-κB, resulting in the dissociation of the inhibitor/NF-κB complex, promoting translocation of NF-κB subunits to the nucleus inducing target gene transcription (53). Notably, we observed a downregulation of IKBKE mRNA expression by CRP in PAEC. IKBKE has been recently identified as a potential key player in orchestrating the balance between innate and adaptive immune systems, suggesting that its down-regulation may result in impaired resistance to infection (36), which could contribute to CTEPH (5).

Additionally, the involvement of an intermediary, which could eventually block transcription of 5-HTR2B, TLR-4/6, and IKBKE genes, such as miRNA, could not be excluded.

**NF-κB partially mediates the effects of CRP on cells of CTEPH patients.** We found that NF-κB mediated the effects of CRP on PAEC, including RelA/NF-κBp65 phosphorylation, adhesion of monocytes to PAEC via ICAM-1 cell surface expression and IL-6 and ET-1 secretion. We have observed...
that NF-κB-specific inhibition by PDTC impaired CRP-induced adhesion of PAEC to monocytes via impaired ICAM-1 expression at PAEC surface, whereas CRP had no effect on other adhesion molecule expression such as VCAM-1 in CTEPH-PAEC (51). c-FOS overexpression results in human umbilical venous EC activation, concomitant to ICAM-1 induction (49). ICAM-1 expression induced by other inflammatory markers, including TNF-α or IL-6, in EC also occurs via NF-κB activation (50). Moreover, the effects of CRP on adhesion molecule expression were investigated by FACS and a significant increase of ICAM expression was observed at the surface of PAEC (data not shown). In addition, focusing on the PCR array findings, a short period of stimulation of 3 h did not induce any de novo ICAM-1 gene expression; consequently, CRP-induced ICAM-1 expression in PAEC is more likely induction of adhesion molecule expression at the cell surface, a well-known feature of EC activation. We also found that CRP-induced IL-6 secretion might be regulated via NF-κB pathway, whereas secretion of monocyte chemotactic protein-1 (MCP-1), a chemokine known to regulate monocyte attraction, was not regulated by CRP. Enhanced IL-6 circulating levels have been observed in CTEPH patients (22). In addition, the IL-6 gene promoter region includes an NF-κB-binding site (27) and ET-1 gene transcription is controlled by NF-κB (41), in accordance with our findings showing an impairment of CRP-mediated ET-1 secretion by NF-κB inhibitor PDTC in PAEC. Transcriptional regulatory mechanisms controlling vWF expression and secretion are restricted to EC and megakaryocytes (15, 46) and remain poorly understood. Common signaling pathways including NF-κB or MAPKinase pathways do not appear to be involved (45). vWF expression and secretion appears to be modulated by distinct signaling pathways involving unknown transcription factors susceptible to interact with different regions of the vWF promoter (14). This could explain the absence of effect of PDTC on CRP-stimulated PAEC.

Relevance of the study. The present study may contribute further elucidating the poorly understood underlying pathological mechanisms in CTEPH by being one of the first studies showing a molecular mechanism potentially involved in the pathophysiology of CTEPH. It further corroborates a potential role of CRP in inducing vascular wall remodeling by showing underlying molecular and intracellular mechanism. Using an inhibitor that directly and specifically impaired the effects of CRP, combined with the observation of CRP-induced RelA/NF-κBp65 phosphorylation, confirmed the involvement of the NF-κB pathway in CRP-mediated effects on PAEC from CTEPH patients. The main results are summarized in Fig. 8.

Limitations of the study. Since the present study specifically focused on the NF-κB pathway, we cannot exclude that other signaling pathways could be involved, especially regarding CRP-induced vWF secretion, which appeared not to be under the control of NF-κB pathway. The absence of various expected upregulated genes and evidence of mainly downregulated genes could be attributed to the fact that PAEC have been

![Diagram](http://ajplung.physiology.org/)

**Fig. 8. Involvement of NF-κB pathway in CRP-induced effects in PAEC in CTEPH.** NF-κB activation by CRP resulted in ICAM-1 expression at PAEC surface, in monocyte adhesion, IL-6 and ET-1 secretion, as shown by their inhibition in the presence of PDTC, an inhibitor of NF-κB signaling. However, NF-κB signaling is not involved in CRP-induced vWF secretion. CRP induced FOS gene expression through NF-κB activation and could also induce ET-1 and IL-6 gene expression since expression of both genes can be regulated by NF-κB (27, 41). Finally, CRP-induced repression of 5-HTR2B, TLR-4, and TLR-6, and inhibitor of NF-κB kinase subunit epsilon (IKBKE) gene expression also involved NF-κB signaling.
isolated from advanced pulmonary vascular lesions, corresponding to a stabilized stage within disease progression or to the short-term (3 h) effects of CRP on PAEC, which implies that the whole machinery usually involved in gene expression regulation has not yet been activated.

Conclusion. We suggest that CRP effects, contributing to persistence of thrombofibrotic material in proximal pulmonary arteries of CTEPH, could be partially attributed to NF-κB pathway activation.

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DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


