Neointimal formation is reduced after arterial injury in human crp transgenic mice

Haim D. Danenberg[a,b,*], **Etty Grad**[a], **Rajesh V. Swaminathan**[b], **Zhiping Chen**[c], **Philip Seifert**[b], **Alexander J. Szalai**[d], **Chaim Lotan**[a], **Daniel I. Simon**[b,c], and **Elazer R. Edelman**[b,c]

[a] Department of Cardiology, Hadassah-Hebrew University Medical Center, 91120 Jerusalem, Israel

[b] Harvard-MIT, Division of Health Sciences and Technology, Cambridge, MA, UK

[c] Cardiovascular Division, Brigham and Women's Hospital, Boston, MA, USA

[d] Division of Clinical Immunology and Rheumatology, The University of Alabama at Birmingham, Birmingham, AL, USA

Abstract

Objectives/Methods—Elevated CRP levels predict increased incidence of cardiovascular events and poor outcomes following interventions. There is the suggestion that CRP is also a mediator of vascular injury. Transgenic mice carrying the human CRP gene (CRPtg) are predisposed to arterial thrombosis post-injury. We examined whether CRP similarly modulates the proliferative and hyperplastic phases of vascular repair in CRPtg when thrombosis is controlled with daily aspirin and heparin at the time of trans-femoral arterial wire-injury.

Results—Complete thrombotic arterial occlusion at 28 days was comparable for wild-type and CRPtg mice (14 and 19%, respectively). Neointimal area at 28d was 2.5 fold lower in CRPtg (4190 ± 3134 μm^2, $n = 12$) compared to wild-types ($10,157 \pm 8890$ μm^2, $n = 11$, $p < 0.05$). Likewise, neointimal/media area ratio was 1.10 ± 0.87 in wild-types and 0.45 ± 0.24 in CRPtg ($p < 0.05$). Seven days post-injury, cellular proliferation and apoptotic cell number in the intima were both less pronounced in CRPtg than wild-type. No differences were seen in leukocyte infiltration or endothelial coverage. CRPtg mice had significantly reduced p38 MAPK signaling pathway activation following injury.

Conclusions—The pro-thrombotic phenotype of CRPtg mice was suppressed by aspirin/ heparin, revealing CRP's influence on neointimal growth after trans-femoral arterial wire-injury. Signaling pathway activation, cellular proliferation, and neointimal formation were all reduced in CRPtg following vascular injury. Increasingly we are aware of CRP multipotent effects. Once considered only a risk factor, and recently a harmful agent, CRP is a far more complex regulator of vascular biology.

Keywords

C-reactive protein; Neointima; Angioplasty; p38 MAPK

*Corresponding author. Tel.: +972 2 6778654; fax: +972 2 6411028. danen@md.huji.ac.il (H.D. Danenberg).

1. Introduction

The acute phase reactant C-reactive protein (CRP) is a powerful predictor for cardiovascular disease on the one hand, and supports an array of vascular biologic events on the other. CRP levels are associated with future cardiovascular events in seemingly healthy subjects and with worse prognosis in acute coronary patients [1]. CRP promotes thrombosis [2,3] and complement activation [4], and might impact lipoprotein metabolism [5]. The confluence of clinical and basic evidence suggests that CRP should predict outcome after vascular intervention. Indeed, several reports have demonstrated a worsened outcome, including high rates of angiographic restenosis, in patients with high pre-procedural CRP levels [6–8]. Others however, found no correlation between CRP and restenosis [9–11]. Dibra et al. reported on the outcome of percutaneous coronary interventions (PCI) in 1152 patients. High CRP levels (>5 mg/l) were not associated with excess restenosis, but myocardial infarction and death at 1 month were twice as prevalent as in patients with lower CRP [10]. Interestingly, such events can commonly be attributed to thrombotic complications. In a second study of more than 900 PCI patients, pre-procedural CRP levels correlated with post-PCI mortality and MI but not with 1-year revascularization, which reflects restenosis [11]. The results of both studies support a causal link between CRP and thrombosis but not with neointimal growth and restenosis.

We have previously shown, using a human CRP transgenic mouse (CRPtg) model, that human CRP accelerates thrombosis [3] induced by guide-wire denudation of the femoral artery or by carotid photochemical injury. The current study examined whether CRP affects neointima formation in the context of PCI independent of its ability to induce thrombosis. Male CRPtg underwent femoral arterial guide-wire injury while receiving drug therapy sufficient to prevent thrombosis. Proliferation and apoptosis were investigated as well as p38 mitogen activated protein kinase (MAPK) activation. Contrary to our expectations, and in contrast to CRP's exacerbation of neointima formation after ligation injury of the carotid artery [12], the guide-wire injured femoral arteries of CRPtg receiving anti-thrombotics had reduced cellular proliferation and neointimal formation as compared to wild-type controls.

2. Methods

2.1. Wire injury model

Male CRPtg and age-matched congenic wild-type mice underwent bilateral wire injury of the femoral artery as described [13]. CRPtg mice are congenic with C57BL/6 and carry a 31-kb *Cla*I fragment of human genomic DNA comprised of the *CRP* gene, 17 kb of 5′-flanking sequence, and 11.3 kb of 3′-flanking sequence [14]. The study was approved by Hebrew University animal ethics committee.

Animals were bled to obtain baseline CRP levels. To prevent thrombosis [3], aspirin treatment was started on the day prior to surgery (5 mg/kg, i.p. daily until artery harvest). A single heparin injection (100 U/kg, i.p.) was administered prior to surgery. All animals received bro-modeoxyuridine (BrdU) (50 mg/kg i.p.) 18 and 1 h before sacrifice. Femoral arteries were harvested for immunohistochemical and morphometric analyses on day 7 ($n = 6$/group) or day 28 after injury ($n = 14–16$/group). These were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into three segments. Multiple sections (5 μm-thick) of each retrieved segment were prepared for VerHoeff tissue elastin and Carstair's fibrin stains, and for immunohistochemical analysis. Microscopic images were captured and the dimensions of media and neointima were determined using Adobe Photoshop 5.0. The one arterial section with maximal neointimal area was chosen for further calculations. Standard avidin–biotin procedures for leukocytes (CD45, DAKO), endothelial cells (CD31,

DAKO) and BrdU were performed. TACS 2 TdT-DAB immunostaining kit (Trevigen) was used for in situ detection of apoptotic cells.

2.2. Signaling pathway activation

Activation of p38 MAPK was measured in the injured arteries 6 h after vascular injury. Pools of 5 arteries from CRPtg mice and their WT littermates were lysed, homogenized under ultrasound, boiled for 5 min and protein content was quantified using the Bradford method. Twenty micrograms of protein were separated in 12% SDS-PAGE gels, blotted onto nitrocellulose and then incubated overnight with primary antibodies specific for mouse p38 (1:500, Cell Signaling) or P-p38 (1:500, Cell Signaling). Activation of p38 MAPK in pooled samples of lung tissue (5 mice per pool) was determined likewise before injury, 6 h after injury and at 24 h after injury. The ratio between the phosphorylated isoforms and the nonphosphorylated isoforms of each protein was calculated.

2.3. Data analysis

Group statistics are presented as the mean $\pm$ S.D. Group means were compared using the unpaired two-tailed Student's t-test. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Arterial response to injury

Serum levels of human CRP in CRPtg, measured by ELISA, were 24 ± 13 and 52 ± 8 mg/l at baseline and at 24 h after injury, respectively, Complete thrombotic occlusion was similar in both groups: observed in 2 of 14 wild-type (14%) and 3 of 16 CRPtg arteries (19%). Wild-type and CRPtg mice not treated with aspirin or heparin bolus had occlusion rates of 15% and 75%, respectively (data not shown), as reported earlier [3]. When mice were treated with aspirin, neointimal formation after guide-wire injury of the femoral artery was less profound in CRPtg compared to wild-type mice (Fig. 1A–C). Intimal area on day 28 was 2.5 fold lower in CRPtg; $10,157 \pm 8890$ μm^2 in wild-type (2136–$22,080$ μm^2, $n = 12$) and 4190 ± 3134 μm^2 in CRPtg (2299–$10,263$ μm^2, $n = 13$, $p < 0.05$). The neointima to media area ratio was similarly lower in CRPtg (1.10 ± 0.87 versus 0.45 ± 0.24, Fig. 1C). The arterial cross-sectional area, i.e. the area within the external elastic lamina, was identical in wild-type and CRPtg mice, $38,802 \pm 11,097$ and $40,444 \pm 10,346$ μm^2, respectively, precluding a major difference in vascular remodeling following injury.

Sections of arteries harvested 7 days after injury were stained for fibrin to confirm the adequacy of thrombotic control. No fibrin was detected in either wild-type or CRPtg mice (not shown). At this time wild-type mice (Fig. 2) demonstrated substantial cell proliferation within the media ($60 \pm 26\%$) and newly formed intima ($13 \pm 7\%$, $n = 5$). These values were 3–4 fold reduced in CRPtg mice ($20 \pm 16\%$ and $3 \pm 2\%$, proliferation in media and intima, respectively, $n = 5$). BrdU incorporation and proliferation at 28 days were sharply reduced to less than 2% in both controls and CRPtg mice. Intimal cell apoptosis at 7 days in CRPtg mice was $1.3 \pm 1.5\%$, more than four-fold reduced when compared to the number of apoptotic cells in wild-type mice ($6 \pm 2\%$, $n = 4$, $p < 0.05$, Fig. 3). In sections obtained at 28 days no apoptotic cells could be detected (not shown). Leukocyte accumulation, assessed by CD-45 immunostaining 7 days after injury, was no different in wild-type and CRPtg mice (16 ± 8 and $14 \pm 11\%$, respectively, $n = 4$, not shown). Endothelialization, evaluated by staining for CD-31 was no different in the arteries of control and CRPtg mice (82 and 77% luminal coverage, respectively data not shown).

3.2. Activation of signaling pathways following injury

The activation of p38 MAPK at 6 h after injury was studied by Western Blot analysis of the phosphorylated and non-phosphorylated isoforms in the injured artery. Proteins were pooled from 5 arteries for each experiment as the amounts of proteins obtained from a single artery were too small to enable such evaluation on their own. Phospho p38 MAPK was reduced in CRPtg mice when compared with wild-type mice. Total p38 MAPK was similar in CRPtg and wild-types, and thus phospho-p38 MAPK normalized to total p38 MAPK remained reduced (Fig. 4, right). The reduction of p38 MAPK activation in CRPtg stands in contradiction to previous studies of this pathway [15]. To further clarify CRP-p38 MAPK inter-action we broadened our studies beyond the injured artery. p38 MAPK activation was studied in lung tissue prior to and at 6 and 24 h after vascular injury. Phopho-p38 MAPK levels, and the ratio between the phosphorylated isoform and total p38 MAPK were reduced at all 3 time points with the largest difference noted at 6 h after injury (Fig. 5, $n = 5$/each time point, $p < 0.05$).

4. Discussion

It is increasingly evident that CRP is not simply a marker of cardiovascular events but a participant in cardiovascular biology. High CRP levels are predictive of acute cardiovascular events and of worse outcome following myocardial infarction and percutaneous coronary interventions, but do not correlate with the burden of atherosclerosis [16,17]. In a transgenic mouse model human CRP clearly predisposed to thrombosis [3], but most reports do not support a causal role in atherogenesis [18–21]. The present study examined the effect of CRP on vascular repair following wire injury in the femoral artery of CRP transgenic mice in which thrombosis was pharmacologically suppressed. In this particular circumstance cell signaling, cell proliferation, and neointima formation were all reduced compared with wild-type controls subjected to the same therapy and injury regimen. This finding is surprising given an earlier report that neointima formation is exacerbated in CRPtg subjected to carotid artery ligation [45], but the two outcomes are not necessarily at odds. Rather, taken together, the results of the two studies might indicate that some or all of the CRP effect on neointima generation might depend on its ability to promote thrombosis, which itself induces cell proliferation. The present study is the first to investigate the influence of CRP on vascular repair in the context of anti-thrombotic therapy.

In vitro data suggest that CRP has vast vascular pro-inflammatory effects (for review [22] icluding production of adhesion molecules, MCP-1 and ET-1 from endothelial cells, blunts endothelial vasoreactivity, reduces NO production and eNOS activity and upregulates the expression of complement inhibitory factors on endothelial cells. Yet, as most of the experiments were in tissue culture with cause–effect exhibited following the use of commercial CRP preparations concern has been raised for spurious effects of contaminants. When impurities, in particular the preservative sodium azide and the contaminant endotoxin, are removed from commercial CRP preparations the putative direct endothelial activity is questioned [23–28]. Transgenic animals that express human CRP, or mice that express rabbit CRP [20], can be used to resolve the issue of this suspected spurious artifactual results. We used CRPtg mice to examine the effects of CRP in vascular injury. This, in spite of less neointima and neointima to media ratio that is observed in mice following vascular injury (as compared with larger species) that warrants larger animals groups to demonstrate discernible differences in repair. CRPtg male mice possess constitutively high and constant CRP expression. CRPtg mice do not reproduce the inflammatory situation observed in high-CRP patients, but can specifically help in studying CRP-associated effects. Consistently elevated levels are achieved without repeated injections that might introduce conformational changes in CRP [29] or contaminants like endotoxin that could affect neointimal formation [30].

In a previous study with CRPtg we found that CRP promotes an arterial pro-thrombotic phenotype [3]. As 75% of the injured arteries in CRPtg mice were occluded by thrombi, further analysis of the effect of CRP on vascular repair was precluded in those studies. To enable such an analysis CRPtg mice were treated with an anti-thrombotic regimen that consisted of heparin at the time of injury, and aspirin beginning a day before injury and continuing for the duration of the study. This empirical regimen suppressed vascular occlusion in CRPtg to the level observed in control wild-type mice. Both heparin [31] and aspirin [32] can provide an anti-proliferative effect in addition to their anti-thrombotic effect. However, suppression of neointimal formation following experimental vascular injury requires substantially higher doses and far longer durations of administration (of heparin) than applied here [32–35]. The decrease in intimal hyperplasia with increased CRP though contrary to expectations may not be surprising in retrospect. While blood CRP is increased in response to inflammatory stimuli, CRP *per se* has anti-inflammatory properties (for review [4,14]). CRP binds and promotes the clearance of apoptotic cells, sustaining an anti-inflammatory response [36]. While apoptosis is generally associated with suppression of neointimal formation [37,38], CRP-induced clearance of apoptotic cells could explain the reduction in apoptotic cells observed in the CRPtg injured vessel. CRP suppresses neutrophil chemotaxis [39] and superoxide generation [40] that are associated with neointimal formation. CRP confers protection against autoimmune diseases: CRPtg are relatively insensitive to murine systemic lupus [41], and experimental allergic encephalomyelitis [42]. Furthermore, CRP upregulated the expression of complement inhibitory factors on endothelial cells [43], suggesting that CRP may protect from complement-mediated vascular injury implicated in intimal hyperplasia [44].

Vascular injury in human CRP transgenic mice on aspirin induced less cellular proliferation, reduced neointimal apoptotic cells and limited neointimal formation. These findings reinforce our earlier proposal that CRP is a potent determinant of cardiovascular biology, but they also reveal that the protein contribution is unexpectedly complex. In the case of neointima formation, the effect of CRP is profoundly influenced by the thrombotic process. The association of high CRP levels with worse outcome after PCI may therefore be derived from vascular pathologies other than proliferation and hyperplasia. Excessive thrombosis alone can induce the pathologies observed in high-CRP patients after vascular intervention [10]. Indeed, the dramatic reduction in thrombotic occlusion after wire injury further supports the extensive use of anti-thrombotics in patients with high-CRP levels. Further studies are warranted to determine whether some drugs are superior to others in suppressing CRP prothrombotic phenotype and if combination therapy should be applied.

The complex cellular interactions initiated by vascular injury are coordinated and modulated by the elaboration of cytokines and growth factors. The production and transduction of many of these mediators require phosphorylation of p38 mitogen-activated protein kinase (MAPK). Activation of p38 MAPK plays an important role in the vascular response, increasing smooth muscle proliferation and neointimal formation. Inhibition of p38 MAPK was previously shown to suppress neointimal formation following vascular injury [45]. The suppressed *in vivo* p38 MAPK activation reported in this study is of special interest. Previous study reported that CRP attains pro-inflammatory activity in peripheral blood mononuclear cells, via the activation of p38 MAPK [15]. Our studies, both in the injured artery and the distant lung tissue, indicate that CRP exerts an opposite effect, with suppression of p38 MAPK. These contradictory findings may result from the *in vitro* methodology which exposes the study to the risk of contamination with preservatives and endotoxin [23–28]. Another explanation is a possible differential effect of CRP on blood mononuclear cells and arterial and alveolar cells. Nevertheless, p38 MAPK activation appears to be reduced in the wounded artery as well as the distant lung and based on the

reported roles of p38 MAPK signaling in vascular reparation we suggest its suppression by CRP as a pathway in the observed CRP anti-proliferative effect.

In the only other comparable study, Wang et al. reported that neointimal formation in the carotid artery of female mice (injured by ligation in mice not given anti-thrombotic therapy) was augmented in CRPtg compared to wild-type [12]. That proliferative effect was suppressed by estradiol administration. The enhanced reparative response observed in that study can be attributed to its contrasting methodology. The current results were obtained using male CRPtg that expressed >15 μg/ml of blood CRP at baseline and underwent denudation of the iliofemoral artery. Due to the high rate of complete thrombotic occlusion mice were treated with an anti-thrombotic regimen. In contrast, no thrombotic occlusion was seen in the carotid artery ligation experiments, but these were performed in females with <0.6 μg/ml of baseline blood CRP. Thus, vascular injury likely was exerted by a different mechanism, injury was induced in different arteries, and CRP blood levels differed by almost two-fold. The enhanced thrombosis that is observed in male CRPtg [3], which is capable of promoting proliferation and neointimal growth and thus was pharmacologically treated was not observed in Wang female mice and was not treated.

4.1. Study limitations

The CRPtg mouse model is devoid of some of the inflammatory changes that are associated with high-CRP levels in human, especially those that stimulate CRP expression such as increased IL-6 levels. Thus, the CRPtg model specifically studies CRP-associated actions, but does not fully reproduce the inflammatory changes observed in high-CRP subjects. Moreover, vascular repair following denudation differs from plaque formation; especially in view of CRP enhanced LDL oxidation [5]. The lesions formed in our model were sufficient to examine the question raised but are clearly relatively simple. Even the extent of endothelial recovery was higher than reported by others [46], perhaps suggesting that we had induced somewhat lesser degree of injury in our model system, but certainly without a difference between our animals or a difference that can itself account for the decreased hyperplasia seen in the CRPtg. Our results remain to be confirmed in more complex animal systems and more injurious vascular injury models such as the ApoE$^{-/-}$ CRPtg model or Watanabe heritable hyperlipidemic rabbits.

In conclusion, following suppression of thrombosis in CRPtg mice neointimal formation after arterial injury was not accelerated but rather decreased. These findings are preceded by suppression of p38 MAPK pro-proliferative signaling, reduced proliferation and reduced number of apoptotic cells. While further studies are called for the role of CRP in vascular repair and atherothrombosis and its predictive value for post-PCI restenosis, the present study suggests a modulating role for CRP on the inflammatory effects of vascular repair. The findings we now report are in line with new data on the multidimensional nature of CRP. Once thought to be only a risk factor, and more recently as only a harmful agent, it is now increasingly understood that CRP is a far more complex regulator of vascular biology.

Acknowledgments

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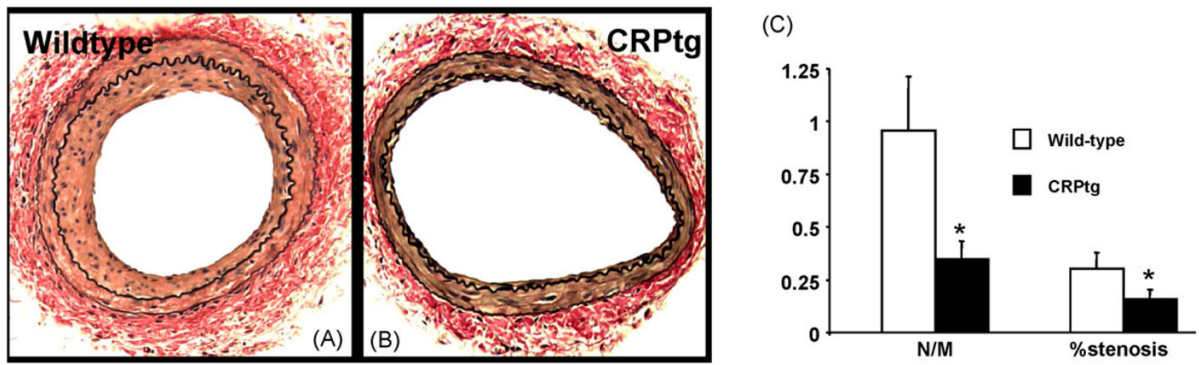
Fig. 1.
Photomicrographs of wild-type (A) and CRPtg (B) femoral arteries stained for elastin at 28 days after wire injury. C, bar graph of neointima to media ratio (N/M) and arterial stenosis in wild-type and CRPtg ($n = 12–13$/group, $p < 0.05$).
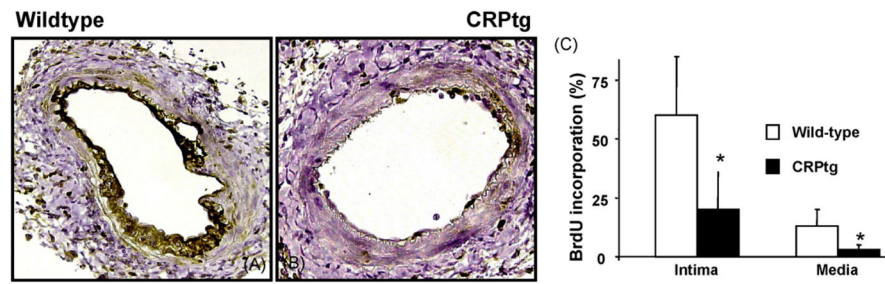
Fig. 2.
Photomicrographs of wild-type (A) and CRPtg (B) femoral arteries stained for and BrdU at day +7 after wire injury. C, bar graph denoting the reduction in the number of proliferating cells in CRPtg compared to wild-type arteries ($n = 5$, $p < 0.05$).
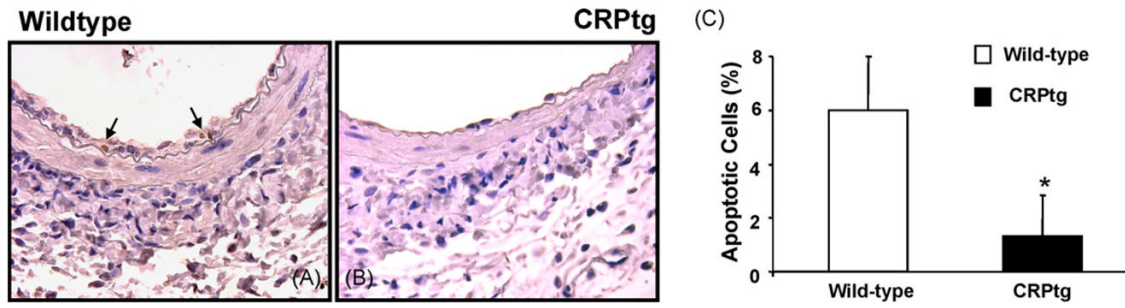
Fig. 3.
Photomicrographs of wild-type (A) and CRPtg (B) femoral arteries stained for apoptotic cells at day +7 after wire injury. C, bar graph denoting the reduction in the number of apoptotic cells in CRPtg compared to wild-type arteries ($n = 4$, $p < 0.05$).
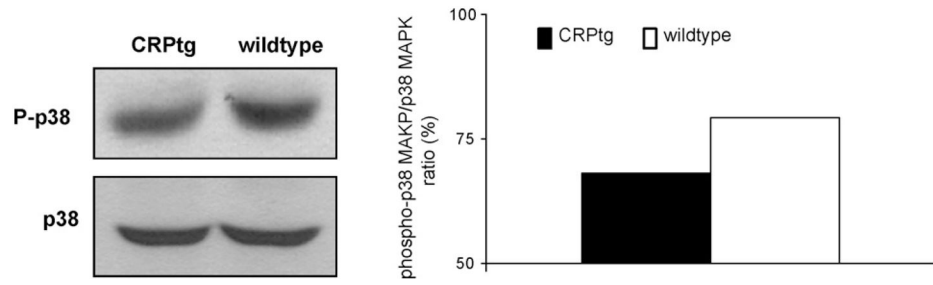
Fig. 4.
Activation of p38 MAPK signaling pathway in the iliofemoral artery at 6 h after wire injury in wild-type and CRPtg mice. On the left panel are representative Western Blots showing the specific bands detected using antibodies against phospho-p38 and p38 MAPK (protein pooled of 5 mice in each Blot). The right panel is a bar graph showing the density ratio between the phopshorylated and total isoforms.
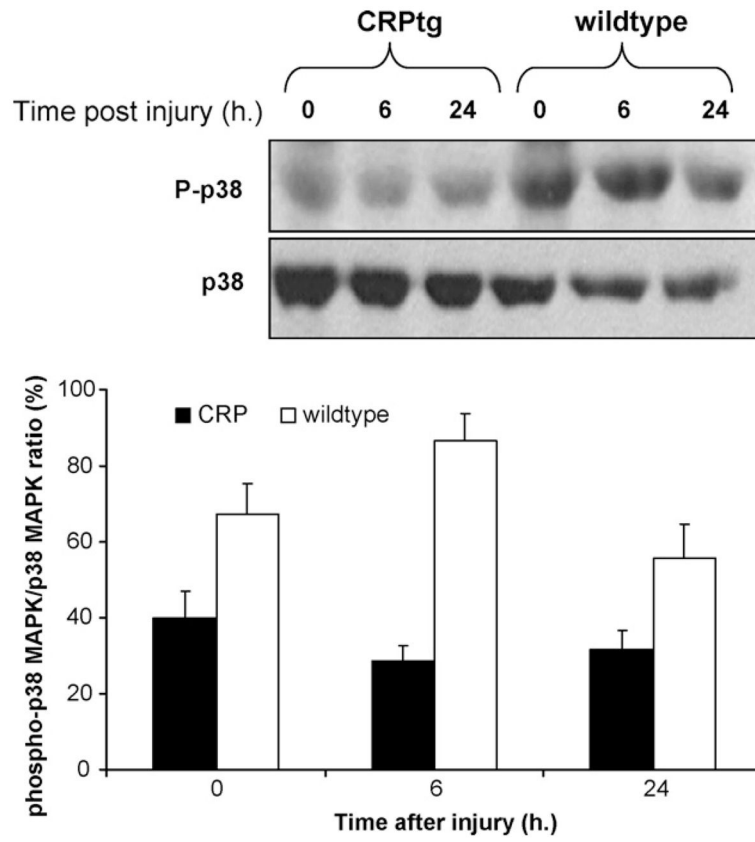
Fig. 5.
Temporal activation of p38 MAPK in the lung prior to and after iliofemoral wire injury. Representative Western Blot showing the specific band detected using antibodies against phosphorylated-p38 and p38 MAPK, and a bar graph showing the density ratio between the phopshorylated and total p38 MAPK isoforms ($n = 5$, mean ratio ± S.D., $p < 0.05$ for all time points).