

IKK β -dependent NF- κ B pathway controls vascular inflammation and intimal hyperplasia

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SPECIFIC AIMS

The aim of the study was to clarify the role of biphasic nuclear factor kappa B (NF- κ B) signaling and I κ B kinase β (IKK β) in the process of vascular repair.

PRINCIPAL FINDINGS

1. Angioplastic injury elicits differential activation of NF- κ B in media and intima

Progression of NF- κ B activation was investigated in rat carotid artery subjected to angioplastic injury. Immunostaining for nuclear NF- κ B p65 showed that the activated NF- κ B signal was present in 55% of medial cells at day 3 postinjury. Later, the signal was located in intimal cells (84%) in conjunction with high levels of IKK activity at day 14. In contrast, a few p65 positive cells and weak IKK activity were detected in media, indicating that NF- κ B activation was resolved at this stage in the media. These results suggest that angioplastic injury elicits two phases of NF- κ B activation characterized by early activation in the media and a later activation in the intima.

2. The early NF- κ B activation underlies the early vascular inflammatory response

To clarify the functional relevance of the early phase of NF- κ B activation, pyrrolidine dithiocarbamate (PDTC) was applied perivascularly to the injured carotid artery via Pluronic F-127 gel, which was rapidly absorbed in vivo by 3 days. The treatment of PDTC suppressed the early NF- κ B activation, as assessed by EMSA, and resulted in 10- and 2-fold reduction in expression of inducible nitric oxide synthase (iNOS) and tumor necrosis factor α (TNF α) at day 3, respectively. The short-term PDTC treatment, however, did not affect intimal formation (Fig. 1).

3. IKK β mediates the late phase of NF- κ B activation and intimal formation

Given the high levels of IKK activity in intima, the specific role for IKK β in vascular repair was elucidated. To this end, endogenous IKK β was disrupted by adenoviral transfection of carotid artery with a mutant form of IKK β (dnIKK β). Overexpression of dnIKK β inhibited the late NF- κ B activation in intima, albeit a lack of effect on the early NF- κ B activation. Overexpression of dnIKK β resulted in down-regulation of iNOS, TNF α , and monocyte chemoattractant protein-1 (MCP-1) expression along with 36% reduction in intima size compared with β -galactosidase gene (β -Gal) transfected vessels at day 14 (Fig. 2).

4. IKK β -dependent NF- κ B signaling is an important mechanism underlying cell proliferation and apoptosis in intima

The present results show that the angioplastic injury induces a potent proliferative response in the media shortly after the injury. Later, the proliferative response was restricted to the intima, characterized by expression of PCNA and cyclin D1 in nearly 80% of intimal cells at day 14. Concomitantly, nearly 30% of cells in the intima underwent apoptosis. Overexpression of dnIKK β exerted dual effects on intimal development, repressing the vascular proliferative response and enhancing apoptosis in intima as assessed at day 14.

CONCLUSIONS AND SIGNIFICANCE

The present study demonstrates that activation of NF- κ B is a prominent response of the arterial wall upon angioplastic injury, and could be defined into two

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Figure 1. Short-term blockade of NF- κ B by PDTC and its impact on vascular inflammatory response and intimal formation. *A*) NF- κ B activation was assessed by EMSA in the injured (control, $n=3$) and the PDTC-treated (PDTC, $n=4$) carotid arteries 3 days postinjury. The specificity of binding was verified by using excess cold NF- κ B or Ap-1 probes. Arrow indicates NF- κ B specific DNA-protein complex. *B*) Transcripts of inducible nitric oxide synthase (iNOS) and tumor necrosis factor α (TNF- α) in injured vessels treated with or without PDTC, as well as in the uninjured vessels, were determined by quantitative real-time RT-PCR (see Materials and Methods). Levels of the transcripts are expressed in arbitrary units normalized to β -actin mRNA. Data are presented as mean \pm SE from 6 to 8 rats for each time point. # $P < 0.05$ vs. uninjured vessels at day 3; * $P < 0.05$ vs. PDTC group at day 3; § $P < 0.05$ vs. uninjured group at day 14. *C*) Histological photographs demonstrate intima thickness of the carotid arteries treated with or without PDTC 2 wk after injury (original magnification: $\times 100$). The arrows denote the most inner layer of internal elastic lamina; arrowheads indicate external elastic lamina, defining the border of intima and media, respectively. The right panel in Fig. 1C shows the quantification of intimal area, medial area, and the ratio of intima to media ($n=5$).

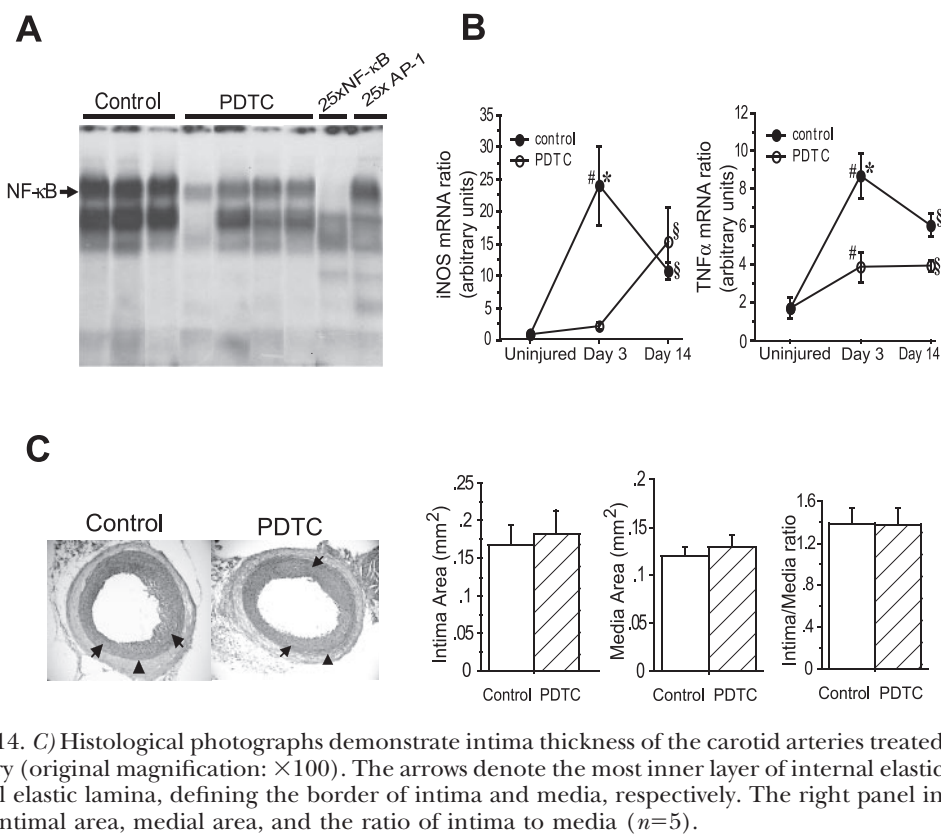
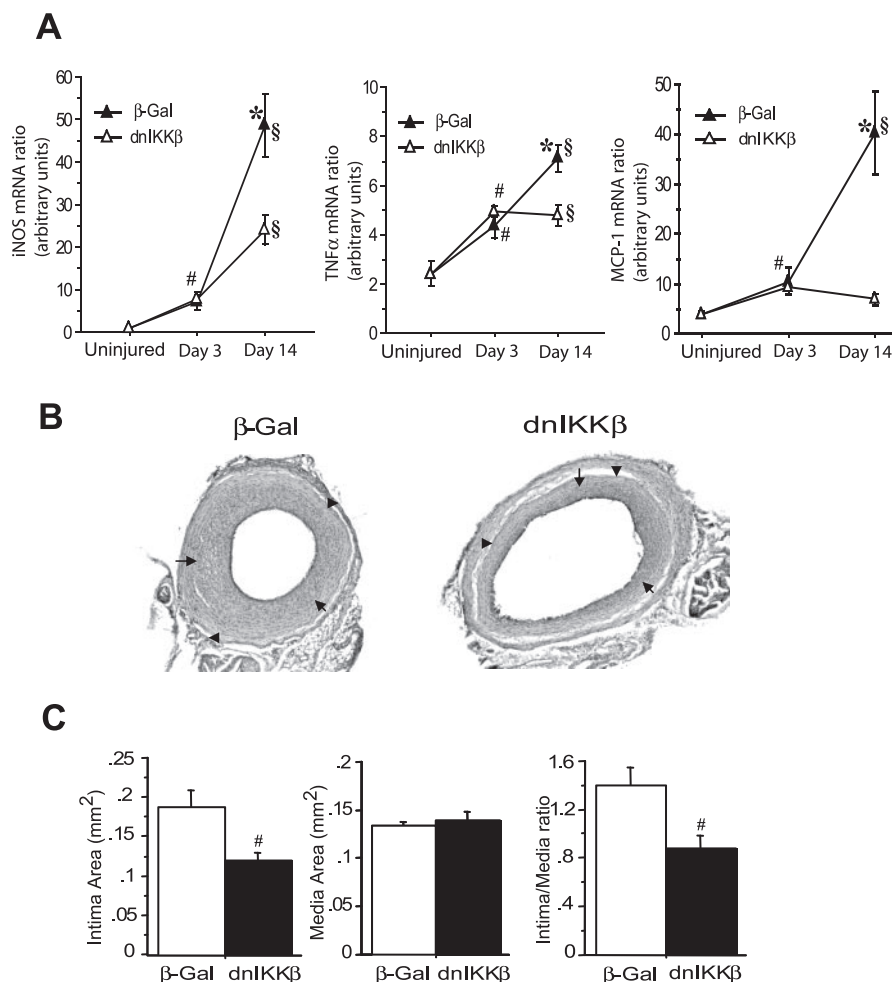


Figure 2. Role of IKK β -mediated NF- κ B signaling in expression of inflammatory genes and intimal formation. *A*) Transcripts of iNOS, TNF α , and monocyte chemoattractant protein-1 (MCP-1) were determined by quantitative real-time RT-PCR (see Materials and Methods). Levels of transcripts are expressed in arbitrary units normalized to β -actin mRNA. Data are presented as mean \pm SE from 6 to 8 rats for each time point. * $P < 0.05$ vs. dnIKK β group at day 14; # $P < 0.05$ in both groups (β -Gal and dnIKK β) vs. uninjured vessels at day 3; § $P < 0.05$ vs. uninjured group at day 14. *B*) Histological photographs demonstrate intima in the carotid arteries transfected with β -Gal or dnIKK β 2 wk after injury (original magnification: $\times 100$). Cross sections from three segments per vessel were examined. Arrows denote the most inner layer of internal elastic lamina, and arrowheads indicate external elastic lamina, defining the borders of intima and the media, respectively. *C*) Quantification of intimal area, medial area, and the ratio of intima to media. $n = 6$ in β -Gal-treated group, and $n = 5$ in the dnIKK β -treated group. # $P < 0.05$ vs. β -Gal.



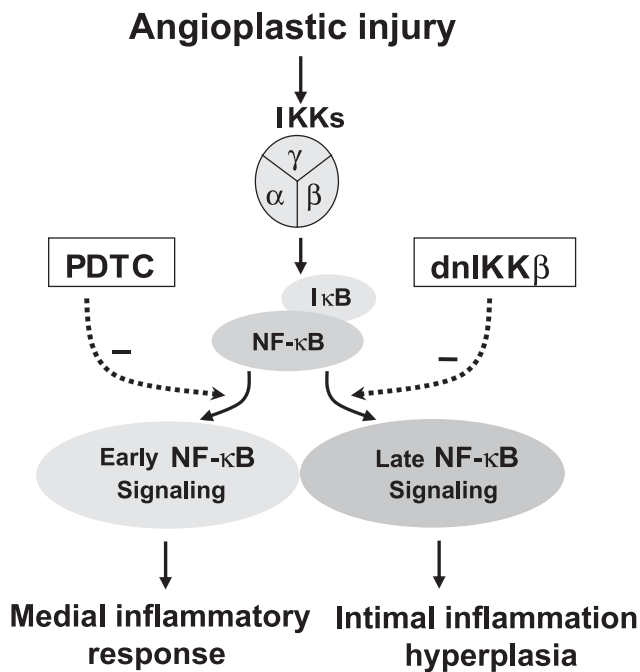


Figure 3. Schematic illustration of angioplastic injury elicited NF- κ B activation and functional relevance in the process of vascular repair. Angioplastic injury induces two differential NF- κ B activations with distinct spatiotemporal features, characterized by an early activation in the arterial media and a late activation coupled with high levels of IKK activity in intima. Blockade by PDTC of the early NF- κ B activation transiently attenuates the expression of proinflammatory genes in the injured vessels, but does not impair the late phase of NF- κ B activation and intimal formation. On the other hand, targeting IKK β by overexpressing a dominant negative IKK β (dnIKK β) in the injured artery effectively inhibits the late phase of NF- κ B activation, resulting in down-regulation of inflammatory gene expression along with marked reduction in intima size. These findings suggest that the IKK β -mediated late phase of NF- κ B activation controls intimal hyperplasia and associated vascular inflammatory responses.

phases based on the distinct temporal and spatial characteristics. As presented in **Fig. 3**, the early NF- κ B activation is involved in the acute inflammatory response in media, but neither the subsequent inflammation nor the hyperplasia in the intima. Interruption of IKK β by overexpression of dnIKK β over a longer postinjury period suppresses the late phase NF- κ B signaling, and concurrently attenuates the vascular

inflammatory response and intimal hyperplasia. These findings indicate that the early and the late NF- κ B activation confer differential functions in the process of vascular repair.

A number of *in vitro* studies suggest that NF- κ B is a crucial regulatory mechanism responsible for inflammatory responses and growth of vascular cells. Nevertheless, the exact role of NF- κ B *in vivo* in the vascular repair process remains debated. The present study suggests that the function of NF- κ B is related to the temporal and spatial pattern of activation. The early NF- κ B activation is elicited in the media upon injury and resolved with development of intima. The function of the transient early NF- κ B signaling is linked primarily with the acute inflammatory response in the media.

The IKK complex is the point of convergence in the cascade of NF- κ B activation. Previous studies suggest that IKK β is 20-fold more active in phosphorylating I κ B α than IKK α and is involved in cytokine-induced activation of the NF- κ B pathway. The current study suggests that IKK β is responsible for the late-phase NF- κ B signaling.

The present data show that IKK β plays an important role in the expression of proinflammatory genes, in particular in the late phase of vascular inflammation. Moreover, IKK β -mediated NF- κ B signaling confers a potent mitogenic activity in intimal formation in part via inducing cyclin D1 and PCNA. In parallel, IKK β -mediated NF- κ B signaling also confers protection against apoptosis of intimal cells *in vivo*. Collectively, these findings highlight IKK β -dependent NF- κ B signaling as the vital mechanism of intimal hyperplasia through regulating vascular inflammation, promoting proliferative responses, and reducing apoptosis.

In summary, the present study provides new insights into the role of NF- κ B activation in vascular inflammation and repair. Our findings show that the early NF- κ B activation is implicated in regulating inflammatory responses in the media, but appears to be dispensable for intimal formation. The late NF- κ B activation mediated by IKK β in the intimal cells is critical in controlling intimal hyperplasia and the associated vascular inflammation. Verifying this assumption would render IKK β in intimal cells an interesting therapeutic target for atherosclerosis and restenosis. **FJ**