Acetylbritannilactone Inhibits Neointimal Hyperplasia after Balloon Injury of Rat Artery by Suppressing Nuclear Factor-κB Activation

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ABSTRACT

Based on our previous observations that 1-O-acetylbritannilactone [(R)-4((3aS,4S,7aR)-4-hydroxy-6-methyl-3-methylene-2-oxo-2,3,3a,4,7,7a-hexahydronaphthacen-5-yl)pentyl acetate (ABL)] suppresses prostaglandin E2 and nitric oxide synthesis in macrophages, the present study was designed to explore the effect of ABL on neointimal hyperplasia after balloon injury and its mechanism of action. In male Sprague-Dawley rats, 26 mg/kg ABL or polyglycol (control) was administered daily from 3 days before injury to 2 weeks after conventional balloon injury. ABL administration led to a significant reduction in neointimal formation (neointima to media ratio, 1.94 ± 0.43 versus 0.84 ± 0.29, P < 0.01) and proliferative activity of vascular smooth muscle cells after balloon injury in rats. Western blot analysis revealed that this is correlated to the inhibition of nuclear factor (NF)-κB activation and to the reduced expression of cyclooxygenase-2. Investigation of potential signaling pathways demonstrated that ABL inhibited NF-κB activation via the blockade of the inhibitor of NF-κB kinase-β activation and the suppression of the degradation of the inhibitors of NF-κB-α. These findings suggest that ABL is a potential inhibitor of neointimal formation because it blocks injury-induced NF-κB activation and may have beneficial effects in reducing the risk of restenosis after angioplasty.

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ABBREVIATIONS: NF, nuclear factor; SMC, smooth muscle cell; ABL, 1-O-acetylbritannilactone, [(R)-4((3aS,4S,7aR)-4-hydroxy-6-methyl-3-methylene-2-oxo-2,3,3a,4,7,7a-hexahydronaphthacen-5-yl)pentyl acetate; PGE2, prostaglandin E2; COX, cyclooxygenase; I, intima; M, media; ELISA, enzyme-linked immunosorbent assay; InB-α, inhibitor(s) of NF-κB-α; IkK, inhibitor of NF-κB kinase; EMSA, electrophoresis mobility shift assay; PCNA, proliferating cell nuclear antigen.
presses nitric oxide and PGE₂ synthesis in macrophages through the inhibition of inducible nitric-oxide synthase and COX-2 gene expression, indicating modulation of inflammation (Han et al., 2004). The present study was designed to elucidate the effect of ABL on neointimal hyperplasia in a model of experimental arterial injury.

Materials and Methods

Animals and Pretreatments. Male Sprague-Dawley rats weighing 290 to 320 g were fed standard laboratory chow and allowed free access to water in an air-conditioned room with a 12-h light/dark cycle. Before the experiment, animals were housed under these conditions for 7 days to allow acclimatization. All animal experiments were approved by the governmental committee for animal research. ABL (99.5% pure), freshly dissolved in 20% polyglycol, was administered orally by gastric gavage from 3 days before balloon injury to 2 weeks after the injury. Six control rats received an equal volume of 20% polyglycol (3 ml/day) in the same manner. Animal body weights were recorded before and at the end of the 2nd week of the experiment.

Balloon Injury Model and after Treatment. Animals were anesthetized with urethane, 600 mg/kg i.p. The thoracic-abdominal artery was de-endothelialized as described previously (Clowes et al., 1983; Capron et al., 1997). In brief, the catheter was advanced from the left common carotid artery down to the level of the renal arteries for 30 mm. The balloon catheter (Boston Scientific Inc. USA, Shelton, CT) was inflated with continuous mixing, and the tube was rocked for 30 min at 4°C. The high-salt buffer consisted of 20 mM HEPES, pH 7.9, 400 mM KCl, 0.2 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol with protease inhibitor cocktail. The nuclear extract was incubated with continuous shaking at 4°C for 30 min and then centrifuged for 15 min at 13,000 g. The supernatants containing the cytosolic proteins were collected. Nuclear protein was extracted by slow addition of 500 µl of high-salt buffer with continuous mixing, and the tube was rocked for 30 min at 4°C. The high-salt buffer consisted of 20 mM HEPES, pH 7.9, 400 mM KCl, 0.2 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol with protease inhibitor cocktail. The nuclear extract was incubated with continuous shaking at 4°C for 30 min and then centrifuged for 15 min at 13,000 g. The supernatants were collected. The protein concentrations of the cytosolic and nuclear extracts were determined using a Quick Start Protein Assay kit (Bio-Rad, Hercules, CA), and then they were aliquoted and stored at −70°C.

Western Blot Analysis. The cytosolic and nuclear proteins (40 µg of protein) were electrophoresed on SDS-polyacrylamide gel and were then transferred onto polyvinylidene difluoride membranes and blotted with specific antibodies against NF-κB p65, IkB-α, glyceraldehyde-3-phosphate dehydrogenase (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), IKK-α, IKK-β, phospho-IKK-α, and phospho-IKK-α/β (Cell Signaling Technology Inc., Beverly, MA). All bands were quantified by a Bio 1D gel analysis system (Vilber Lourmat, Marne-la-Vallée, France). The molecules in the bands of interest were visualized by autoradiography.

Determination of Proliferative Activity. To determine whether suppressed inflammation is accompanied by the reduced proliferative activity of SMCs, we performed immunohistochemical staining against proliferating cell nuclear antigen (PCNA; Santa Cruz Biotechnology, Inc.), and the percentages of PCNA-positive cells versus total nucleated cells were quantified in three different sectors per tissue section (n = 6 in each group).

Statistics. All of the experiments were repeated at least three times with a similar pattern of results. Data are expressed as the mean ± S.D., and the ABL treatment effects were analyzed by Student’s t test using SPSS 11.0 software (SPSS Inc., Chicago, IL). A value of P < 0.05 was considered to be statistically significant.

Results

Body Weight and Plasma Level of ABL. The mean body weight was not affected by treatment with ABL at 14 days after balloon injury (control group, 298 ± 7 g; ABL-treated group, 298 ± 7 g). The maximum plasma concentration of ABL was reached within 1 to 2 h of drug administration, which was 0.17 ± 0.03 mg/ml (n = 6).

ABL Inhibits Neointimal Hyperplasia. At 14 days after balloon injury, the control animals showed abundant neointimal hyperplasia (Fig. 2C). The ABL-treated animals showed significant suppression of neointimal hyperplasia and reduction of the I/M ratio (control versus ABL, 1.94 ± 0.08 versus 1.23 ± 0.07; P < 0.05).
ABL Inhibits the Production of PGE₂ by Suppressing Injury-Induced COX-2 Expression. To test whether the inhibiting neointimal hyperplasia effect of ABL is related to its anti-inflammatory properties, the effects of ABL on COX-2 gene expression and the production of PGE₂ were examined. Western blot analysis showed that rat arteries uninjured by the balloons produced modest signals, indicating that sham operations might stimulate COX-2 expression to some extent. Balloon injury resulted in high levels of COX-2 expression on day 14 in the control group. In ABL-treated rats, the expression of COX-2 induced by balloon injury was significantly reduced. Densitometric analysis showed that ABL reduced the COX-2 band intensity by 65.7 ± 7.9% (Fig. 3A). However, COX-2 was not modified directly by ABL (data not shown). Moreover, balloon injury resulted in a clear increase in the serum PGE₂ level, which was reduced by 63.7 ± 9.4% on day 14 after balloon injury in ABL-treated rats (control versus ABL, 2600 ± 195 versus 945 ± 136 pg/ml, n = 6; *, P < 0.01) (Fig. 3B). In addition, we observed a positive correlation between the serum PGE₂ level and COX-2 expression 14 days after the injury (Pearson correlation coefficient, 0.947; n = 12; P < 0.001, two-tailed) (Fig. 3C).

ABL Suppresses NF-κB Activation by Inhibiting IκB-α Phosphorylation and Nuclear Translocation of p65. We found that the injury-induced expression of p65 started to increase at day 1 after balloon injury and reached
Fig. 4. ABL suppressed NF-κB activation and activity. A, effects of balloon injury on p65 protein expression in arteries at different time points. n = 6 per time point per group. B, ABL markedly attenuated injury-induced p65 protein expression at 14 days. C, NF-κB-DNA binding activity 14 days after balloon injury. The results indicated that ABL inhibited NF-κB activation in nuclear extracts of injured rat arteries. The specificity of binding was verified by using 100-fold excess cold NF-κB probes. Arrows, NF-κB-specific DNA-protein complexes. The densitometry analyses of NF-κB protein-DNA complex for the corresponding EMSA assessments are shown. D, correlation between I/M ratio and NF-κB-DNA binding activity on day 14. Data shown were obtained as described in Figs. 2E and 4C. These results demonstrated that ABL treatment inhibits NF-κB-DNA binding activity, which is associated with neointimal formation (n = 12, Pearson correlation coefficient = 0.940, P < 0.001). E, ABL inhibited the nuclear translocation of p65. Western blot analysis showed ABL suppressed the translocation of p65 into the nucleus at 14 days after balloon injury. F, ABL suppressed IκB-α degradation and phospho-IκB-α on day 14. At the bottom, densitometry analyses of Western blot are provided as the mean ± S.D. of three separate experiments. *, P < 0.01 versus injured group. n = 6 in each group.
the peak level at day 14 (Fig. 4A). To explore the effect of ABL on p65 expression, Western blot was performed on arterial samples at 14 days after balloon injury. At this time point, the p65 expression was reduced by 42.9 ± 5.7% in the ABL-treated group compared with the injured group (Fig. 4B).

To investigate whether NF-κB is a significant target for the anti-inflammatory action of ABL in balloon injured animals, we examined the effect of ABL on NF-κB activation by performing an EMSA. The results, presented in Fig. 4C, showed a low level of NF-κB-DNA binding activity was detected in nuclear protein extracts from arteries of control rats. Conversely, a strong NF-κB p65-specific gel-retarded band was detected in samples derived from balloon-injured animals (Fig. 4C, lane 3). The binding of NF-κB p65 is DNA-specific because the band disappeared in the presence of an excess of unlabeled NF-κB oligonucleotides (Fig. 4C, lane 5). In contrast, faint gel-retarded bands were found in samples from ABL-treated animals (Fig. 4C, lane 4). Furthermore, we also performed a Pearson correlation analysis to determine whether ABL-induced suppression of neointimal hyperplasia resulted from the reduced NF-κB activity. Our analysis (shown in Fig. 4D) indicated a positive correlation between the DNA-binding activity of NF-κB and the I/M ratio 14 days after balloon injury (Pearson correlation coefficient, 0.940; n = 12; P < 0.001, two-tailed).

Nuclear translocation of the Rel family protein p65, as a marker for NF-κB activation, was also determined. As shown in Fig. 4E, balloon injury stimulated the translocation of p65 into the nucleus. However, ABL treatment suppressed the translocation of the cytosolic p65 subunit to the nucleus in the injured arteries of ABL-treated rats on day 14. The effect of ABL on the degradation of IκB-α was also examined. At this time point, balloon injury resulted in a significant increase in IκB-α degradation, whereas this effect was significantly blocked by ABL treatment. Meanwhile, the level of phosphorylated IκB-α was reduced to 15.0 ± 3.6% in comparison with the injured group (Fig. 4F).

ABL Inhibits the Expression and Phosphorylation of IKK-β. To elucidate the molecular mechanisms of the ABL-limited neointimal hyperplasia, further experiments were conducted to explore the pathway by which ABL diminishes the inflammatory response induced by the balloon injury. Phosphorylation of IKKs is a critical step and a point of convergence in the NF-κB activation pathways (Karin, 1999; Karin and Ben-Neriah, 2000). ABL completely blocked the expression of IKK-β triggered by balloon injury on day 14 but did not affect that of IKK-α. Then, we examined the effects of ABL on IKK phosphorylation. Likewise, the phosphorylation of IKK-β was dramatically attenuated by ABL. Interestingly, the phosphorylation level of IKK-α was unaltered (Fig. 5). Thus, it was speculated that ABL inhibited IκB-α phosphorylation by specifically suppressing that of IKK-β.

Effect of ABL on the Proliferative Activity of SMCs. Suppressed inflammation was accompanied by a reduction in the proliferative activity of SMCs. We stained sections for PCNA expression in representative fields from each artery and determined the percentage of PCNA-positive nuclei in the neointima. The number of PCNA-positive cells in the neointimal and medial layers of the arteries remarkably decreased in ABL-treated rats 14 days after injury, compared with the control animals (Fig. 6, C and D). At day 14, the PCNA-positive percentage in controls was 36.4 ± 9.8%, whereas in the ABL-treated group, it was 14.9 ± 4.2% (P < 0.01).

Discussion

The activation of the inflammatory response to vessel injury has been shown in numerous studies to play a predominant role in the development of postangioplasty restenosis (Bu et al., 2005; Chandrasekar et al., 2006). Vessel injury is associated with the induced expression of the proinflammatory genes, including vascular cell adhesion molecules, cytokines, growth factors, and proinflammatory enzymes, which are the mechanisms leading to SMC proliferation, vessel wall remodeling, foam cell accumulation, and neointimal hyperplasia.

ABL is a natural substance, extracted from the traditional Chinese medicinal herb I. britannica L. In vitro studies have suggested that the anti-inflammatory effect of ABL involves blocking the binding of NF-κB to the promoter in the target genes and inhibiting the expression of the proinflammatory genes by suppressing lipopolysaccharide-induced IκB degradation in macrophages (Han et al., 2004). In the present investigation, the effects of ABL on NF-κB activation, an important mediator of inflammation, cell proliferation, and restenosis, were studied. Our results indicate that administration of ABL in vivo leads to a significant reduction in neointimal thickness by ~56.7% (Fig. 2) 2 weeks after angio-
plasty. The study demonstrates that ABL is a potent inhibitor of NF-κB activation by specifically suppressing the phosphorylation of IKK-β with consequent inhibition of NF-κB translocation and of IκB degradation. Furthermore, we observed a positive correlation between NF-κB activation and the degree of neointimal hyperplasia.

NF-κB is a crucial transcription factor driving the inflammatory response (Tak and Firestein, 2001; Chen and Shi, 2002; Raines et al., 2004). NF-κB dimers are sequestered in the cytoplasm in an inactive form associated with regulatory proteins called IκB. Inflammatory stimuli such as lipopolysaccharide, interleukin-1, and tumor necrosis factor-α cause phosphorylation of IκB-α and its subsequent degradation, allowing translocation of NF-κB to the nucleus, where it binds to the promoters of NF-κB regulatory genes and initiates transcription of the proinflammatory gene (Chen et al., 1995; Scherer et al., 1995; Ghosh et al., 1998; Huxford et al., 1998; Hoffmann et al., 2002). The evidence that inflammation mediated by NF-κB is involved in postangioplasty lumen narrowing suggests that NF-κB plays a causative role in the development of restenosis (Bu et al., 2005; Chandrasekar et al., 2005). The fact that NF-κB/IκB-α complexes shuttle constitutively between the cytoplasm and the nucleus provides us with a new idea that the regulation of this important signaling pathway is a prospective clue to preventing a potential inflammatory response (Birbach et al., 2002; Kitamoto et al., 2003; Tsatsas et al., 2006). Thus, the suppression of NF-κB-dependent inflammatory gene expression may be an extremely effective therapeutic strategy for preventing inflammatory processes and neointimal growth (Kitamoto et al., 2003).

It has been reported that the use of antisense oligonucleotides to the p65 subunit of NF-κB could reduce neointimal formation by inhibition of SMC proliferation and adherence (Autieri et al., 1995). Moreover, studies using gene delivery to overexpress IκB-α have been shown to be effective in suppressing neointimal formation in a rabbit restenosis model (Breuss et al., 2002). These studies, together with the present results, point toward NF-κB as an attractive therapeutic target for intimal thickening. Supporting the role of the inhibitory effect of ABL on inflammation as a causal mechanism was the suppression of IκB-α phosphorylation and the activation of NF-κB. These were confirmed by EMSA and Western blot. ABL treatment reduced NF-κB-DNA binding activity by 43.4 ± 7.9% (Fig. 4C) and the IκB-α phosphorylation by 85.1 ± 3.6% (Fig. 4F). To elucidate the molecular mechanisms leading to the inhibition of NF-κB activation by ABL, we explored the local expression of IKK in injured arteries. The IKK complex is the point of convergence in the cascade of NF-κB activation. Given the potent IKK activity in intima and the role for IKKβ-mediated NF-κB signaling in inflammation, we further provide experimental evidence that ABL specifically inhibits phosphorylation of IKK-β triggered by balloon injury (Fig. 5). These findings are consistent with previous data from the literature indicating that IKK-β represents a key target of various NF-κB inhibitors, including manumycin A and peroxynitrite (Levrand et al., 2005; Bernier et al., 2006). Although ABL blocked IKK-β phosphorylation, it did not affect IKK-α phosphorylation. This indicates that there may be a unique mechanism by which ABL specifically inhibits phosphorylation of IKK-β, which requires further investigation to acquire more evidence. Taken together, these results indicate that the effect of ABL may be dependent on the phosphorylation of IKK-β.

In the present study, we measured the serum PGE₂ level, which is tightly coupled with the COX-2 protein expression that increased after balloon injury in vivo. It has been reported that prostaglandins might influence the development of restenosis and intimal hyperplasia (Connolly et al., 2002). PGE₂ enhanced platelet aggregation, chemotaxis of leukocytes, vascular permeability, and vascular cell adhesion molecule expression, thus resulting in serious damage in the arterial walls (Roviezzo et al., 2005; Chen et al., 2006; Kamachi et al., 2007). Therefore, we believe that not only local inflammations but also systemic inflammations, as measured by serum PGE₂, play an important part in the development of neointimal hyperplasia. Such findings are supported by other studies, which showed that nonspecific systemic inflammation can aggravate neointimal hyperplasia after balloon injury in the rat (Park et al., 2004). This interpretation is consistent with the positive correlation between the reduced blood serum PGE₂ level and the down-regulated expression of COX-2 protein (Fig. 3C). In this study, we found that ABL suppressed the local expression of COX-2 significantly, which not only affects PGE₂ biosynthesis but also reduces the vascular inflammatory response. We assume in our studies that the reduced level of PGE₂ might...
be responsible for the decreased risk of restenosis. However, we observed that ABL did not have a significant modification on COX-2. Thus, the decrease in PGE2 level by ABL resulted from inhibition of COX-2 expression. It is possible to hypothesize that circulating PGE2 synthesis may be induced from sources other than the injured arteries, and inhibition of NF-κB activation by ABL may cause a decreased expression of other molecules, which is the downstream of NF-κB and related to the COX-2 gene expression.

It is well established that SMCs are the major cellular component of neointimal lesions, and proliferation of SMCs plays an important role in the pathogenesis of restenosis. PCNA is synthesized in early G1 and S phases of the cell cycle. ABL diminishes the number of PCNA-positive cells in the neointima (Fig. 6). These findings suggest that ABL may suppress inflammatory neointimal hyperplasia after balloon injury not only by anti-inflammation but also by inhibition of SMC proliferation. The inhibition of proliferation may also contribute to the reduced levels of inflammatory proteins and transcription factors found in lesions of ABL-treated rats. All of these effects of ABL may be beneficial in the prevention of neointimal hyperplasia.

In conclusion, this is the first study to demonstrate that ABL inhibits neointimal hyperplasia after angioplasty. The inhibition is associated with the modulation of vascular inflammation and inhibition of SMC proliferation via attenuation of IKK-NF-κB signaling. Besides providing novel insights into the protective action of ABL in the vascular injury response, these results offer a potential therapeutic strategy of ABL in the prevention and treatment of cardiovascular inflammatory diseases and restenosis after angioplasty.

References


