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Beneficial effects of caffeic acid phenethyl ester in a rat model of vascular injury

[1]Pasquale Maffia, [2]Angela Ianaro, [2]Barbara Pisano, [2]Francesca Borrelli, [2]Francesco Capasso, [1]Aldo Pinto & *,[2]Armando Ialenti

[1]Department of Pharmaceutical Science, University of Salerno, via Ponte Don Melillo Fisciano, Salerno, Italy and [2]Department of Experimental Pharmacology, University of Naples 'Federico II', via Domenico Montesano, 49-80131 Naples, Italy

1 The aim of this study was to evaluate whether caffeic acid phenethyl ester (CAPE), an active component of propolis, was able to reduce neointimal formation in a model of endothelial injury of rat carotid artery (balloon angioplasty). Furthermore, we investigated the relationship between neointima formation and nuclear factor-κB (NF-κB) activation and we correlated NF-κB activation to the expression of inducible isoform of cyclo-oxygenase (COX-2) in injured carotids.

2 In control group a significant proliferation of neointima was observed 14 days after balloon angioplasty, which was correlated to an increase of NF-κB/DNA binding activity as well as p50/p65 nuclear levels compared to those observed in the carotids from sham-operated rats. Furthermore, NF-κB activation was correlated to increased COX-2, but not β-actin, protein expression.

3 Treatment of rats for 14 days with CAPE (3, 10, 30 mg kg^{-1}) caused a significant inhibition of all the parameters assayed, except β-actin protein expression.

4 These results indicate that treatment with CAPE may lead to a reduction of neointima formation by inhibiting NF-κB activation and suggest that this agent may have therapeutic relevance for the prevention of human restenosis.

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Keywords: Balloon angioplasty; CAPE; cyclo-oxygenase-2; nuclear factor-κB; restenosis; vascular injury

Abbreviations: CAPE, caffeic acid phenethyl ester; COX-2, inducible cyclo-oxygenase; DNA, deoxyribonucleic acid; EDTA, ethylenediaminetetraacetic acid; EMSA, electrophoretic mobility shift assay; IEL, internal elastic lamina; iNOS, inducible nitric oxide synthase; MCP-1, monocyte chematactic protein-1; mRNA, messenger ribonucleic acid; NF-κB, nuclear factor-κB; PBS, phosphate-buffered saline; PEG, polyethyleneglycol; PTCA, percutaneous transluminal coronary angioplasty; SDS, sodiododecylsulfate; TUNEL, terminal uridine nick-end labelling

Introduction

Percutaneous transluminal coronary angioplasty (PTCA) is a widely used technique in the therapy of coronary artery stenosis where the occluded segment is dilated by inflating a balloon catheter. One of the major problems related to this technique is the re-narrowing of the vessel, a phenomenon known as restenosis which occurs in 30–40% of patients within 3–6 months (Ferrell *et al*., 1992). The principal animal model that has been used to study this process is the response of the normal rat carotid artery to balloon angioplasty (Ross, 1993). Balloon-induced vascular injury, which includes disruption of both the endothelial barrier and medial smooth muscle layer, brings about a cascade of events involving platelets activation, thrombin generation and the release of mitogens that results in the final hyperplastic response shown by the neointima (Ip *et al*., 1991). Intimal hyperplasia, which is partly responsible for restenosis, results from migration of smooth muscle cells across the internal elastic lamina followed by their elevated proliferation in the intima of the rat artery (Schwartz *et al*., 1995).

It is well known that the expression of several genes involved in the inflammatory and proliferative responses of cells are regulated at transcriptional level by the nuclear factor-κB (NF-κB) (Baeuerle, 1991). The activation of NF-κB has been found to occur in balloon-injured rat carotid arteries and has been associated to neointima formation and expression of NF-κB regulated genes, including adhesion molecules and pro-inflammatory enzymes such as the inducible isoforms of both nitric oxide synthase (iNOS) and cyclo-oxygenase (COX-2) in injured vessels (Ialenti *et al*., 2001; Landry *et al*., 1997). We have already shown that an *in vivo* treatment with the antioxidant agent pyrrolidine dithiocarbamate reduced neointimal formation in balloon-injured rat carotid arteries, an effect associated to inhibition of NF-κB activation (Ialenti *et al*., 2001).

Propolis is a natural substance, produced by honeybees from the gum of various plants. It contains several chemical compounds, including various phenolic compounds like flavonoids (galangine, quercetin), cinnamic acid and its derivatives such as caffeic acid phenethyl ester (Burdock, 1998). It has been demonstrated that caffeic acid phenethyl ester (CAPE) is a potent and specific inhibitor of NF-κB activation (Natarajan *et al*., 1996). CAPE has antiviral, anti-inflammatory and immunomodulatory properties and it has been shown to inhibit the growth of different types of transformed cells (Grunberger *et al*., 1988; Mirzoeva & Calder, 1996; Su *et al*., 1994).

*Author for correspondence; E-mail: ialenti@unina.it

In this study we investigated whether CAPE would influence neointima formation after balloon injury of the rat carotid artery. Furthermore, the *in vivo* inhibition of NF-κB activation has been correlated to the expression of COX-2 in the rat carotid.

Methods

Animals

Male Wistar rats (Harlan, Italy) weighing 275–300 g were used for this study. Animals were housed in propylene cages with food and water *ad libitum*. The light cycle was automatically controlled (on 0700 h; off 1900 h) and the room temperature thermostatically controlled to $22 \pm 1^{\circ}$C. Prior to the experiment animals were housed in these conditions for 4–5 days to become acclimatized.

Balloon angioplasty

Animals were anaesthetized with an intraperitoneal injection of ketamine (100 mg kg^{-1}) and xylazine (5 mg kg^{-1}). Angioplasty of the right carotid artery was performed according to the procedure described by Indolfi *et al*. (1995) by using a balloon embolectomy catheter (2F, Fogarty; SEDA, Italy). The balloon catheter was introduced through the right external carotid artery into the aortic arch and inflated at two atmospheres with a calibrated inflation device (Indeflator Plus 20, Advanced Cardiovascular System, Inc). The carotid artery was damaged by passing the inflated balloon catheter back and forth through the lumen three times (control group). CAPE (3, 10, 30 mg kg^{-1}), freshly dissolved in polyethyleneglycol (PEG), was administered orally, by gastric gavage, once a day for 14 days. Control animals received an equal volume of PEG (0.4 ml rat^{-1}). Some animals were subjected to anaesthesia and surgical procedure without balloon injury (sham-operated rats). Rats were sacrified 14 days after vascular injury and carotid arteries were removed and processed as described below.

Morphology

After 14 days from angioplasty animals were anaesthetized as described above and the carotid arteries were fixed by perfusion with 100 ml of phosphate-buffered saline (PBS; pH 7.2) followed by 80 ml of PBS containing 4% paraformaldehyde through a large cannula placed in the left ventricle. The carotid arteries were removed, and six cross sections were cut (each 6 μm thick) from the approximate midportion of the artery, with three of the sections stained with haematoxylin and eosin to demarcate cell types. The remaining three sections were stained with aldehyde fuchsin and counterstained with van Gieson's solution to demarcate the internal elastic lamina (IEL). The sections were photographed under low power, videodigitized, and stored in the image analysis system (Qwin Lite 2.2, Leica). The media, neointima and vessel wall were traced carefully, and the neointima/media ratio was calculated. The carotid sections in which IEL was ruptured were rejected.

TUNEL method

The terminal uridine nick-end labelling (TUNEL) method was used to evaluate apoptosis at balloon injury sites according to the procedure described by Malik *et al*. (1998) by using an apoptosis detection kit (Appligene Oncor) with the chromogen diaminobenzidine. The counterstain was 0.5% methyl green. When viewed with a $\times 40$ objective, cells showing morphologic features characteristic of apoptosis in addition to positive TUNEL reaction were considered to be apoptotic. Non specific cytoplasmic staining without nuclear involvement was considered negative. At least 500 nuclei were counted from each slides. Staining results were expressed as percentage of TUNEL-positive cell nuclei.

Macrophage infiltration

Carotid sections were deparaffinized, endogenous peroxidase was quenched with 0.3% H_2O_2 in 60% methanol for 30 min. Non-specific adsorption was minimized by incubating the section in 1% BSA in phosphate-buffered saline for 40 min. Sections were subsequently incubated for 1 h at 37°C with the primary monoclonal antibody anti-ED1 (1 : 100 dilution), which is specific against rat monocytes and macrophages. Sections were then washed in PBS containing 1% BSA three times before incubation with biotinylated secondary antibody and labelling with peroxidase avidin/biotin complex, with 3.3′-diaminobenzidine used as the chromogen. The mean percentage of macrophages was calculated as the per cent of ED1^{+} positive cells in relation to the total number of cells in the neointima and media area.

Preparation of cytosolic fractions and nuclear extracts

The carotid segments were frozen in liquid nitrogen, immediately suspended in 1 ml of ice-cold hypotonic lysis buffer (mM): HEPES 10, $MgCl_2$ 1.5, KCL 10, phenylmethylsulphonylfluoride 0.5, 1.5 μg ml^{-1} soybean trypsin inhibitor, 7 μg ml^{-1} pepstatin A, 5 μg ml^{-1} leupeptin, 0.1 mM benzamidine, 0.5 mM dithiothreitol, and homogenized at the highest setting for 1 min in a Polytron PT 3000 (Kinematica AG) tissue homogenizer. The homogenates were chilled on ice for 15 min and then vigorously shaken for 15 min in the presence of 25 μl of 10% Nonidet P-40. The nuclear fraction was precipitated by centrifugation at $1500 \times g$ for 5 min. The supernatant containing the cytosolic fraction was collected, and protein concentration was determined by the Bio-Rad (Milan, Italy) protein assay kit, then it was aliquoted and stored at -80°C until use. The nuclear pellet was resuspended in 600 μl of high salt extraction buffer (mM): HEPES pH 7.9 20, NaCl 420 mM, $MgCl_2$ 1.5 mM, ethylenediaminetetraacetic acid (EDTA) 0.2, 25% v v^{-1} glycerol, 0.5 mM phenylmethylsulphonylfluoride, 1.5 μg ml^{-1} soybean trypsin inhibitor, 7 μg ml^{-1} pepstatin A, 5 μg ml^{-1} leupeptin, 0.1 mM benzamidine, 0.5 mM dithiothreitol, and incubated under continuous shaking at 4°C for 30 min. The nuclear extract was then centrifuged for 15 min at $13{,}000 \times g$. The supernatant was collected, protein concentration was determined by the Bio-Rad (Milan, Italy) protein assay kit, then it was aliquoted and stored at -80°C.

Electrophoretic mobility shift assay (EMSA)

EMSA studies were performed on nuclear extracts as previously described (Iuvone *et al.*, 1998). In brief, double stranded NF-κB consensus oligonucleotide probe (5′-AGTTGAGGGGATTTTCCCAGGC-3′; synthesized by CEINGE, Naples, Italy) was end-labelled with ^{32}P-γ-ATP. Nuclear extracts (15 μg protein for each sample) were incubated for 30 min with radiolabelled oligonucleotides ($2.5-5.0\times10^4$ c.p.m.) in 20 μl reaction buffer containing 2 μg poly dI-dC, 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1 μg μl^{-1} bovine serum albumin, 10% (v v^{-1}) glycerol. In competition reaction nuclear extracts from injured carotid arteries were incubated with radiolabelled NF-κB probe in absence or presence of identical but unlabelled oligonucleotides (W.T. 50×), mutated non-functional κB probe (Mut. 50×) or unlabelled oligonucleotide containing the consensus sequence for Sp-1 (Sp-1 50×). In supershift experiments nuclear extracts from injured carotid arteries were incubated with antibodies against p50, p65, p50 + p65 or c-Rel 30 min before incubation with radiolabelled NF-κB probe. Nuclear protein-oligonucleotide complexes were resolved by electrophoresis on a 5% non denaturing polyacrylamide gel in 0.25×TBE buffer at 150 V for 2 h at 4°C. The gel was dried and autoradiographed with intensifying screen at −80°C for 20 h. Subsequently, the relative bands in nuclear fractions were quantified by densitometric scanning of the X-ray films with a GS 700 Imaging Densitometer (Bio-Rad) and a computer program (Molecular Analyst, IBM).

Immunoprecipitation and Western blot analysis

The levels of p50, p65 and β-actin and the expression of COX-2 and β-actin were quantified in nuclear and cytosolic extracts respectively by immunoprecipitation followed by Western blot analysis according to the manufacturer's instructions (Santa Cruz Biotechnology, Milan, Italy). Briefly, equivalent amounts of either nuclear or cytosolic extracts (200 μg for each sample) were mixed 40 μl of protein A-sepharose and 2 μl of anti-p50, anti-p65, anti-β-actin and anti-COX-2 antibodies and left overnight at 4°C with continuous shaking. Immunocomplexes were washed three times with 500 μl of buffer A (10 mM TRIS-HCl pH 7.5, 1 M NaCl, 0.2% Triton X-100 and 2 mM EDTA), mixed with 40 μl of gel loading buffer (50 mM Tris/10% sodiododecylsulfate (SDS)/10% glycerol/10% 2-mercaptoetanol/2 mg of bromophenol per ml) and then boiled for 3 min. Samples so obtained were electrophoresed in a 12% discontinuous polyacrylamide minigel. The proteins were transferred onto nitrocellulose membranes, according to the manufacturer's instructions (Bio-Rad). The membranes were saturated by incubation at 4°C overnight with 10% non-fat dry milk in PBS and then incubated with anti-p50, anti-p65, anti-β-actin or anti-COX-2 antibodies for 1 h at room temperature. The membranes were washed three times with 1% Triton X-100 in PBS and then incubated with anti-rabbit or anti-goat immunoglobulins coupled to peroxidase. The immunocomplexes were visualized by the ECL chemiluminescence method (Amersham, Milan, Italy). Subsequently, the relative bands were quantified by densitometric scanning of the X-ray films with GS-700 Imaging Densitometer (Bio-Rad, Milan, Italy) and a computer program (Molecular analyst, IBM).

Statistic

Data are expressed as means ± s.e.mean of (n) rats. Statistical significance was calculated by one-way analysis of variance (ANOVA) and Bonferroni-corrected P value for multiple comparisons. The level of statistically significant difference was defined as $P<0.05$.

Reagents

Phosphate-buffered saline was from Celbio (Milan, Italy). DL-dithiothreitol, phenylmethylsulphonylfluoride, soybean trypsin inhibitor, pepstatin A, leupeptin and benzamidine were from Calbiochem (Milan, Italy). ED1 was from Serotec (Milan, Italy). ^{32}P-γATP was from ICN Biomedicals (Milan, Italy). Poly dI-dC was from Boehringer-Mannheim (Milan, Italy). p50-, p65-, c-Rel- and COX-2-specific anti-peptide antibodies were from Santa Cruz Biotechnology (Milan, Italy). Non-fat dry milk was from Bio-Rad (Milan, Italy). All other reagents were from Sigma (Milan, Italy).

Results

Effect of CAPE on neointimal proliferation

Representative cross-sections of the carotids arteries from sham-operated rats, control rats subjected to balloon injury and rats treated with CAPE (30 mg kg^{-1}) subjected to balloon injury are shown in Figure 1. In sham-operated rats ($n=6$) not subjected to vascular injury, no neointimal formation was observed while in control group ($n=10$) neointima area was of 0.220 ± 0.007 mm^2 (Figure 2). CAPE at the dose 3 mg kg^{-1} ($n=7$) did not modify neointima area, which was reduced by 16% ($n=8$) and 42% ($n=8$; $P<0.01$) when animals were treated with 10 and 30 mg kg^{-1} CAPE, respectively (Figure 2). No treatments affected the media area (Figure 2).

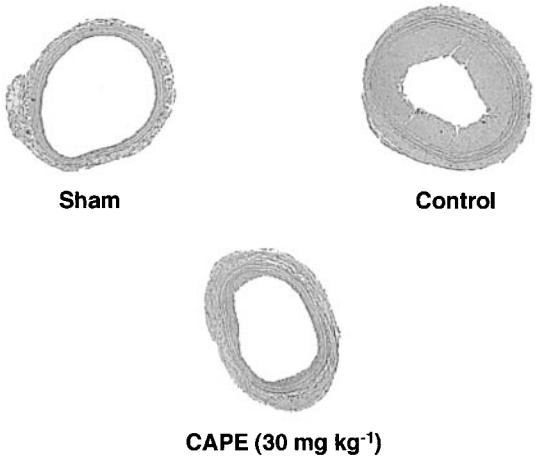

Figure 1 Photomicrographs showing the neointimal formation in rat carotid arteries 14 days after balloon injury. Representative cross sections are from the carotid artery of sham-operated rat, control rat subjected to balloon injury and rat treated with CAPE (30 mg kg^{-1}) subjected to balloon injury.

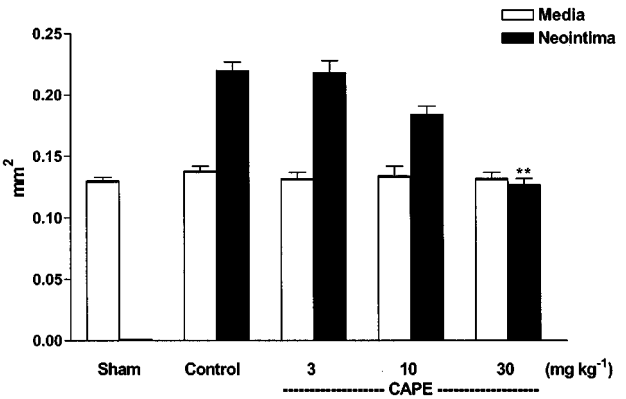

Figure 2 Effect of CAPE (3, 10, 30 mg kg^{-1}) on media (open bars) and neointima (solid bars) areas of injured rat carotid arteries. Results are expressed as mean ± s.e.mean. $^{**}P<0.01$ *vs* control group.

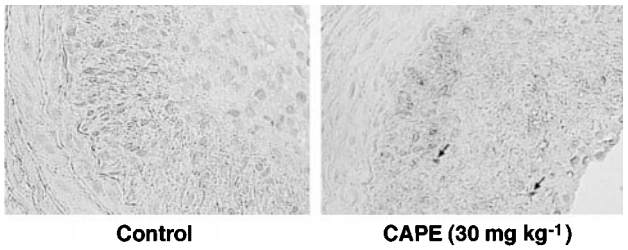

Figure 3 Detection of apoptosis 14 days after balloon injury. TUNEL-positive cells are seen within neointima only of injured carotids arteries from rats treated with CAPE (30 mg kg^{-1}). No TUNEL-positive cells were seen in control group.

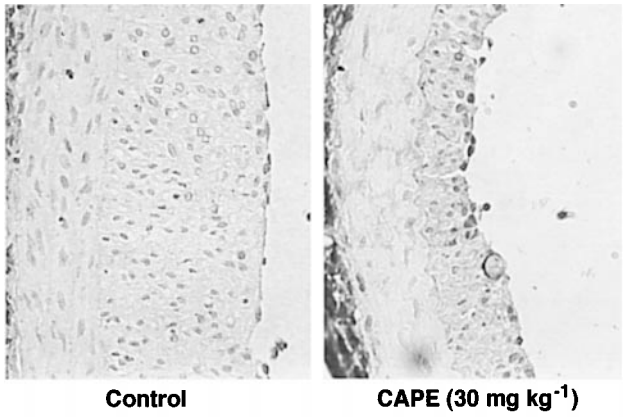

Figure 4 Representative photomicrographs of sections of carotid arteries at 14 days after balloon angioplasty labelling for the macrophage antigen ED1.

Effect of CAPE on apoptosis

Apoptosis was not detected in control vessels at 14 days after balloon angioplasty (Figure 3). TUNEL-positive cells were detectable within neointima only of injured carotids from rats treated with 30 mg kg^{-1} CAPE (4.2 ± 0.3%, $n=5$).

Effect of CAPE on macrophage infiltration

In sham-operated rats, a mean of 1.6 ± 0.4% of macrophages were found in the intimal layer (data not shown). In control group a significant infiltration of $ED1^+$ cells (32 ± 3%) was observed within neointima at 14 days after balloon angioplasty (Figure 4). Macrophage infiltration was significantly inhibited by about 20% ($P<0.05$; $n=5$) in carotids from rats treated with 30 mg kg^{-1} CAPE.

Effect of CAPE on NF-κB activation

A low level of NF-κB/DNA binding activity was detected in nuclear protein extracts from carotid arteries of sham-operated rats. Conversely a retarded band was clearly shown in injured carotid arteries from control rats. As shown by densitometric analysis NF-κB/DNA binding activity was inhibited by CAPE (30 mg kg^{-1}) by 46% ($n=3$; $P<0.01$) (Figure 5).

Characterization of NF-κB complex

The specificity of the NF-κB/DNA binding complex was evident by the complete displacement of the NF-κB/DNA binding in the presence of a 50 fold molar excess of unlabelled NF-κB probe (W.T. 50×) in the competition reaction (Figure 6). In contrast, a 50-fold molar excess of unlabelled mutated NF-κB probe (Mut. 50×) or Sp-1 oligonucleotide (Sp-1 50×) had no effect on these DNA binding activity. The composition of the NF-κB complex activated by angioplasty was determined using specific antibodies against p50 (anti-p50), p65 (anti-p65) and c-Rel (anti-c-Rel) subunits of NF-κB proteins (Figure 6). Addition of the anti-p65 led to decreased complex formation that implicated inhibition of the DNA-binding ability of the p65 NF-κB subunit after antibody addition as previously shown (Cercek *et al*., 1997; Newton *et al*., 1997; von Knethen *et al*., 1999). In contrast, addition of anti-p50, as well as anti-p50 + anti-p65, shifted the complex to a higher molecular weight. The addition of anti-c-Rel to the binding reaction did not result in a marked reduction of NF-κB band intensity.

Effect of CAPE on p50, p65 and β-actin nuclear levels

In control animals high levels of both p50 and p65 NF-κB subunits were detected in injured carotids by immunoblotting analysis as compared to sham-operated rats (Figure 7A,B). Densitometric analysis showed that CAPE (30 mg kg^{-1}) significantly reduced both p50 (by 40%, $n=3$, $P<0.01$) and p65 (by 42%, $n=3$, $P<0.01$) band intensity. The expression of β-actin was analysed by Western blotting for comparative purposes. In carotids from sham-operated rats as well as control animals the expression of this protein remained unchanged. Furthermore, the β-actin expression was not affected by treatment of rats with CAPE (30 mg kg^{-1}) (Figure 7C).

Effect of CAPE on COX-2 and β-actin protein expression

A significant level of COX-2 protein expression was detected in injured carotids arteries of control animals compared to sham-operated rats (Figure 8A). Densitometric analysis showed that CAPE (30 mg kg^{-1}) reduced COX-2 band intensity (by 47%, $n=3$, $P<0.01$). Furthermore, in carotids from sham-operated rats as well as control animals the expression of β-actin remained unchanged. The expression of

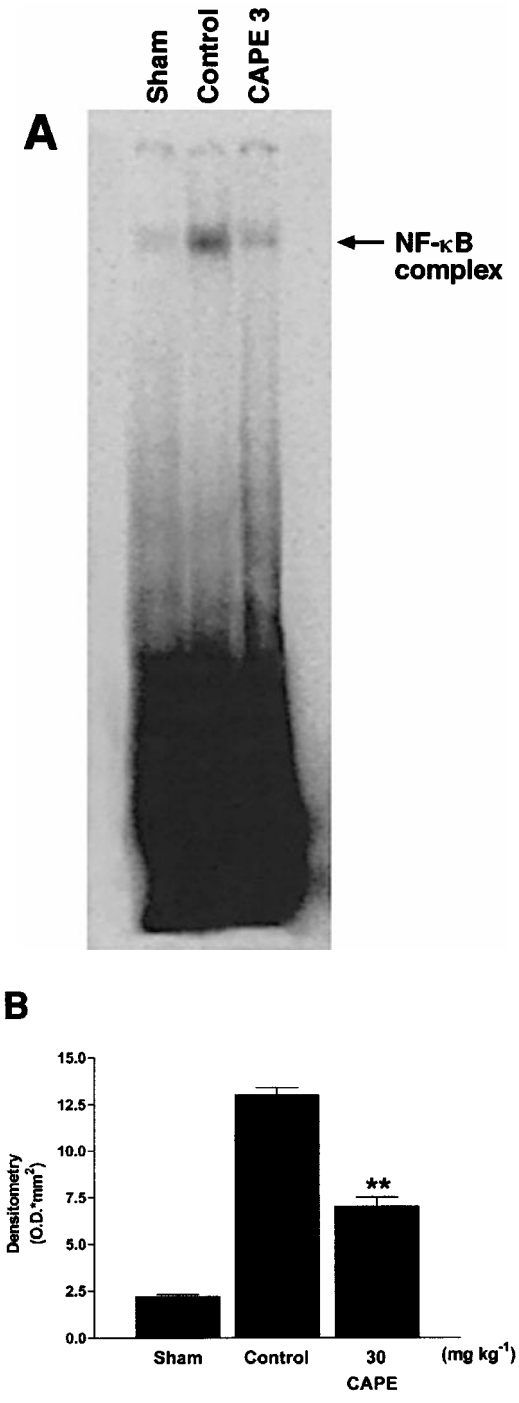

Figure 5 Representative electrophoretic mobility shift assay of NF-κB (A) and densitometric analysis (B) showing the effect of CAPE (30 mg kg^{-1}) on NF-κB/DNA binding activity in injured rat carotid arteries. Results are expressed as mean$\pm$s.e.mean of three separate experiments. $^{**}P<0.01$; *vs* control group.

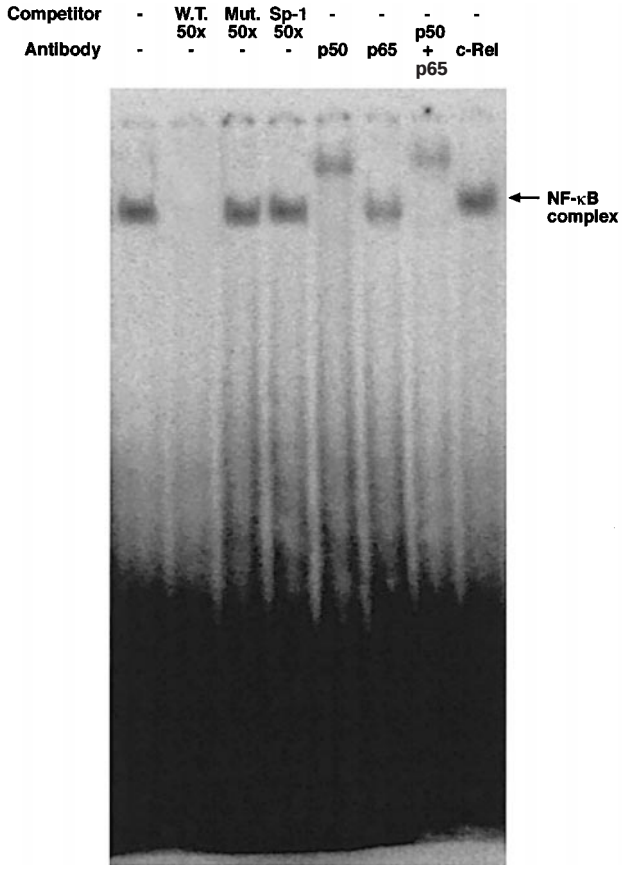

Figure 6 Identification of NF-κB/DNA binding proteins in nuclear extracts of injured rat carotid arteries. Nuclear extracts were prepared as described in the text and incubated with ^{32}P-labelled NF-κB probe. In competion reaction nuclear extracts were incubated with radiolabelled NF-κB probe in the absence or presence of identical but unlabelled oligonucleotides (WT. 50$\times$), mutated non functional κB probe (Mut. 50$\times$) or unlabelled oligonucleotide containing the consensus sequence for Sp-1 (Sp-1 50$\times$). In supershift experiments nuclear extracts were incubated with antibodies against p50, p65 or c-Rel 30 min before incubation with radiolabelled NF-κB probe. Results illustrated are from a single experiment and are representative of three separate experiments.

this protein was not affected by treatment of rats with CAPE (30 mg kg^{-1}) (Figure 8B).

Discussion

The results of the present study show that *in vivo* administration of CAPE is able to inhibit neointima formation in balloon-injured rat carotid arteries. This inhibition is correlated to an inhibition of NF-κB activation and to a reduced expression of COX-2.

The most studied NF-κB complex is a heterodimer of two subunits, p50 and p65, that is present in the cytoplasm in inactive form associated with an inhibitory subunit IκB-α (Baeuerle, 1991). The phosphorylation of IκB-α and its subsequent degradation allows translocation of NF-κB to the nucleus where it binds to the promoters of NF-κB regulated genes and initiates gene transcription (Baeuerle & Henkel, 1994; Thanos & Maniatis, 1995).

The activation of NF-κB in the vessel wall after arterial injury in rats and mice has been established by several reports and has been correlated to the induced expression of NF-κB-dependent genes including vascular cell adhesion molecules, cytokines, growth factors and pro-inflammatory enzymes such as iNOS and COX 2 (Cercek *et al*., 1997; Ialenti *et al*., 2001; Landry *et al*., 1997). In samples of rat injured carotids we detected both p50 and p65 subunits, but not cRel, suggesting that the complex NF-κB in rat arteries might

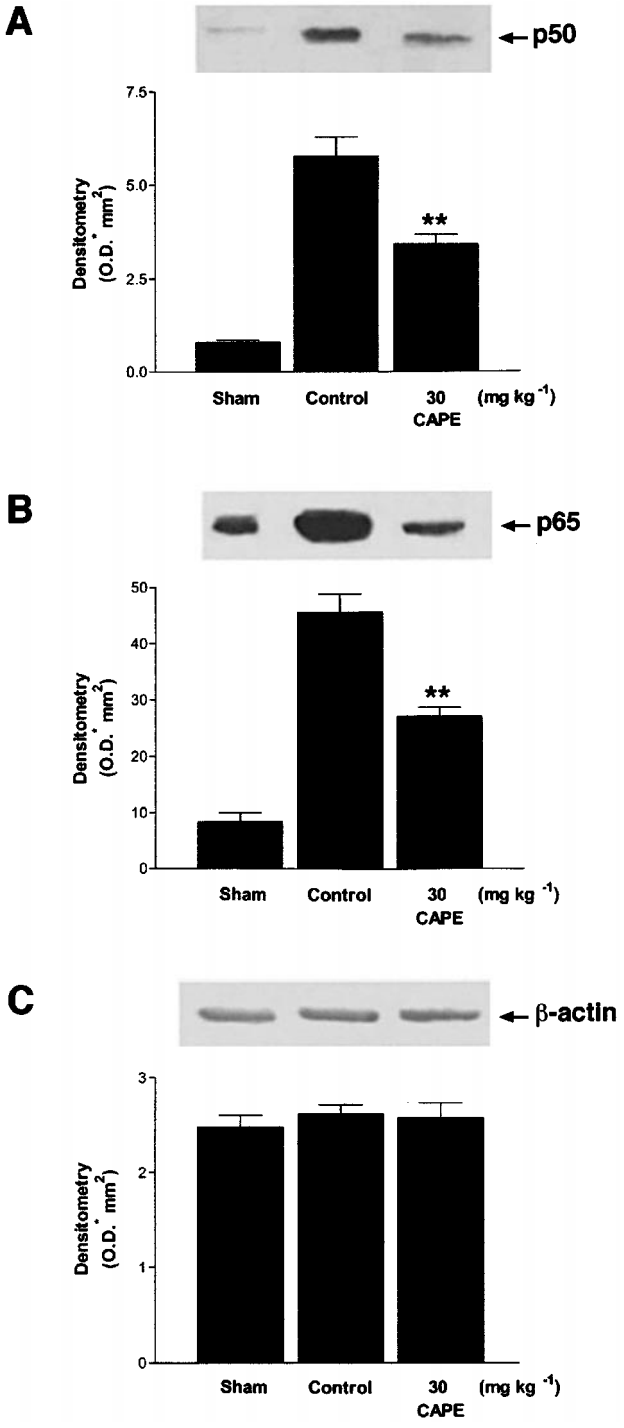

Figure 7 Representative Western blot and relative densitometric analysis showing the effect of CAPE (30 mg kg^{-1}) on p50 (A), p65 (B) and β-actin (C) nuclear levels in injured carotid arteries. Results are expressed as mean $\pm$ s.e.mean of three separate experiments. $^{**}P<0.01$ *vs* control group.

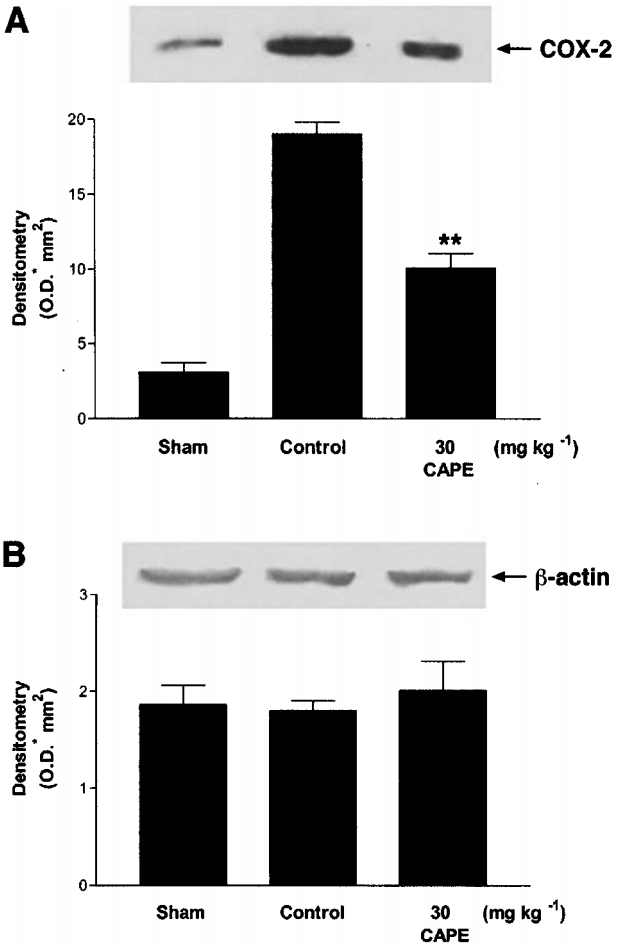

Figure 8 Representative Western blot and relative densitometric analysis showing the effect of CAPE (30 mg kg^{-1}) on COX-2 (A) and β-actin (B) protein expression in injured rat carotid arteries. Results are expressed as mean $\pm$ s.e.mean of three separate experiments. $^{**}P<0.01$ *vs* control group.

result from the association of these heterodimeric forms as already shown by Cercek *et al.* (1997). NF-κB is an attractive therapeutic target for the pharmacological control of vascular response to injury. It has been shown that the use of antisense oligonucleotide to the p65 subunit of NF-κB inhibited neointima formation (Autieri *et al.*, 1995). Consequently the development of NF-κB inhibitors with therapeutic efficacy is widely recognized and in this light CAPE could represent an interesting molecule.

CAPE has been shown to be a pharmacologically safe compound with anti-inflammatory, immunomodulatory, anticarcinogenic and anti oxidant properties. It has been shown that CAPE completely blocked the activation of NF-κB induced by a wide variety of inflammatory agents (Natarajan *et al.*, 1996). However is not clear how CAPE inhibits the activation of NF-κB. The activation of NF-κB in injured carotids may depend on the formation, by endothelial cells, smooth muscle cells and/or infiltrating leukocytes, of several mediators including large amounts of reactive oxygen intermediate species (Babbs *et al.*, 1991; Griendling *et al.*, 1994; Morris *et al.*, 1995) which have been shown to release the inhibitory subunit IκB-α (Schmidt *et al.*, 1995). Consequently it is possible that CAPE exerts its effects by inhibiting reactive oxygen intermediates production (Bhimani *et al.*, 1993; Laranjinha *et al.*, 1995). The inhibition of NF-κB activation by CAPE may also be ascribed to its ability to alter the redox state of the cell (Chiao *et al.*, 1995). Interestingly it has been reported that CAPE induces apoptosis in several cells lines (Chiao *et al.*, 1995; Orban *et*

al., 2000). Our results indicate that apoptosis, evaluated by using TUNEL method, occurs in neointima of the injured carotids of rats treated with CAPE and, consequently, the induction of apoptosis may represent an additional inhibitory mechanism of neointima formation by CAPE.

Furthermore, in this study we observe that the reduction in the degree of restenosis elicited by CAPE is also accompanied to reduced macrophage deposition in injured vessels. The mechanism by which CAPE affect macrophage infiltration remain unknown. However, it is possible to hypothesize that inhibition of NF-κB activation may cause a decreased expression of adhesion molecules, including monocyte chemotactic protein-1 (MCP-1), a potent and specific chemokine for monocytes/macrophages, whose gene expression is induced by NF-κB (Landry *et al*., 1997).

In this study we have also shown that NF-κB inhibition resulted in a reduced expression of COX-2, whose gene is NF-κB-regulated, whereas β-actin expression remained unaffected, showing that the treatment with CAPE actually inhibited molecular events downstream NF-κB activation. This finding is in agreement with results showing that CAPE suppressed the induction of COX-2 messenger ribonucleic acid (mRNA) expression both in cultured human epithelial cells and in rat carrageenin air pouch model of inflammation (Michaluart *et al*., 1999). We did not measure the amounts of prostanoids produced by injured carotids, therefore we are unable to establish whether the reduced expression of COX-2 was associated to a substantial reduction in the biosynthesis of prostanoids. The role of prostanoids in balloon angioplasty is not clear since it has been demonstrated that only high doses of aspirin, i.e. having an inhibitory effect on NF-κB activation, are able to reduce neointimal thickening following balloon injury in rat (Cercek *et al*., 1997). This may explain the lack of the effect of current clinical doses of aspirin on experimental and clinical restenosis (Topol & Serruys, 1998).

Conclusions

In conclusion, the results of our study show that an *in vivo* treatment with CAPE, an active component of propolis, inhibited NF-κB activation, along with the expression of NF-κB-regulated enzyme COX-2, and reduced neointimal formation in balloon-injured rat carotid arteries, suggesting that this compound may be useful for the pharmacological control of vascular response to injury and for the prevention of human restenosis.

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