

# A novel mechanism for the inhibition of NF- $\kappa$ B activation in vascular endothelial cells by natural antioxidants<sup>1</sup>

SHAY Y. SCHUBERT, ISHAK NEEMAN,\* AND NITZAN RESNICK<sup>†,2</sup>

The Interdepartmental Program in Biotechnology, \*Faculty of Food Engineering and Biotechnology,

<sup>†</sup>Department of Anatomy and Cell Biology, Bruce Rappaport Research Institute and the Rappaport Faculty of Medicine, Technion, Haifa, Israel

## SPECIFIC AIMS

Many vascular diseases result from or are accompanied by inflammatory processes, where activation of nuclear factor  $\kappa$ B (NF- $\kappa$ B) plays a pivotal role. In the present study we tested the ability of a natural antioxidant, pomegranate wine, to inhibit NF- $\kappa$ B activation in vascular endothelial cells comparing it to that of red wine and N-acetyl cysteine (NAC) and defined a novel mechanism for this inhibition, a mechanism that does not involve I $\kappa$ B $\alpha$  phosphorylation and stabilization but interferes with the level of NF- $\kappa$ B (p65) phosphorylation.

## PRINCIPAL FINDINGS

### 1. Inhibition of NF- $\kappa$ B activation by RW, PW, and NAC in EC

It has long been demonstrated that RW (and more recently, PW) can scavenge free radicals and inhibit oxidation both in vitro and in vivo. Little is known of the ability of PW and RW to act as antioxidants in vascular endothelial cells. To test the antioxidant activity of RW and PW in situ, arterial EC cultures were pretreated with the antioxidants for 1 h before simultaneous addition of H<sub>2</sub>DCFDA (5  $\mu$ M) and TNF- $\alpha$  (200 U/mL). Treatment of TNF- $\alpha$  alone resulted in excessive oxidation of H<sub>2</sub>DCF over control untreated cultures. Quantification of the data (4 fields for each condition, of the ratio fluorescent cells/total cells, using Image-pro 4 software) revealed a fourfold increase in TNF-treated cells over control untreated cells, implying for the formation of ROS. Cultures pretreated with antioxidants RW or PW (2  $\mu$ g/mL) demonstrated up to 20-fold less oxidation of H<sub>2</sub>DCF than cultures treated with TNF- $\alpha$  alone and up to 4-fold less oxidation compared with untreated cultures, indicating a strong in situ antioxidant activity of both agents.

To test the activity of PW, RW, and NAC as NF- $\kappa$ B

inhibitors, BAEC cultures (passages 3–6) were pretreated with RW or PW (4  $\mu$ g/mL) for 2 h and exposed to TNF- $\alpha$  (200 U/mL) for 5–40 min. Nuclear and cytosolic fractions were tested for the presence of p65. TNF- $\alpha$  treatment alone resulted in a rapid (5 min) p65 nuclear translocation whereas pretreatment with RW, PW, or NAC inhibited this migration (2- to 4-fold). Inhibition of p65 nuclear translocation by these antioxidants was accompanied by inhibition in p65 binding activity as revealed by EMSA (data not shown). NF- $\kappa$ B inhibition by these antioxidants was also observed when endothelial cells were exposed to a biomechanical stimulus (physiological levels of laminar shear stress, 10 dynes/cm<sup>2</sup>) for 30 min. Reduced levels of p65 in the nucleus after antioxidant treatment were not the result of changes in expression of the molecule. We further examined three isolated compounds from RW and PW reported as bioactive antioxidants—quercetin, catechin, and gallic acid—for their ability to inhibit p65 nuclear translocation in TNF- $\alpha$ -treated cell. All three compounds significantly inhibited TNF- $\alpha$ -induced p65 translocation, gallic acid being the most potent inhibitor.

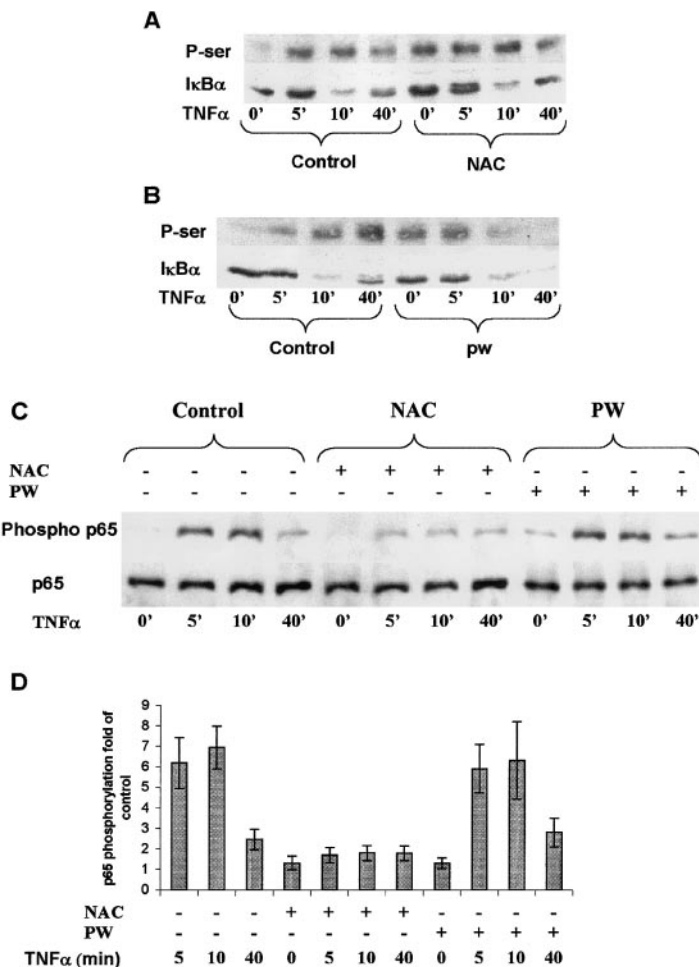
### 2. Pretreatment with PW or NAC does not affect I $\kappa$ B $\alpha$ phosphorylation or degradation in TNF- $\alpha$ -treated BAEC

Treatment of endothelial cells with TNF- $\alpha$  leads to a rapid serine phosphorylation of I $\kappa$ B $\alpha$  (Fig. 1A, B), followed by rapid degradation of the molecule (Fig. 1A, B). Whole cell extracts were prepared from untreated BAEC cultures treated with TNF- $\alpha$  or antioxidants before addition of TNF- $\alpha$  and tested for

<sup>1</sup> To read the full text of this article, go to <http://www.fasebj.org/cgi/doi/10.1096/fj.02-0147fje>; to cite this article, use *FASEB J.* (October 4, 2002) 10.1096/fj.02-0147fje

<sup>2</sup> Correspondence: Department of Anatomy and Cell Biology, Bruce Rappaport Research Institute, Bruce Rappaport Faculty of Medicine, Efron St., P.O. Box 9697, Bat-Galim, Haifa, Israel. 31096. E-mail: nitzanr@tx.technion.ac.il

**Figure 1.** Treatment with PW and NAC does not inhibit TNF- $\alpha$ -mediated I $\kappa$ B $\alpha$  phosphorylation and degradation, but selectively inhibits p65 phosphorylation. BAEC were treated with NAC (20 mM) (A) or PW (2  $\mu$ g/mL) (B) for 2 h before incubation with TNF- $\alpha$  (200 U/mL) for 5, 10, and 40 min. Whole cell extracts were prepared and tested for serine phosphorylation of I $\kappa$ B $\alpha$  or its degradation ( $n=4$ ). BAEC were treated with NAC (20 mM) or PW (4  $\mu$ g/mL) (C) for 2 h before incubation with TNF- $\alpha$  (200 U/mL) for 5, 10, and 40 min. Whole cell extracts were prepared and tested for ser536 phosphorylated (a) or unphosphorylated (b) p65. Quantitative analysis (D) of the blots was carried out using scanning densitometry ( $n=6$ ). Statistically significant inhibition of p65 phosphorylation by NAC, but not PW, in TNF-treated cells is observed.



serine phosphorylation of I $\kappa$ B $\alpha$  with an anti-phospho-serine antibody (Fig. 1A, B). In TNF- $\alpha$ -treated cultures, I $\kappa$ B $\alpha$  serine phosphorylation was evident as early as 5 min after the treatment. To our surprise, pretreatment of BAEC with either PW or NAC before exposure to TNF- $\alpha$  did not result in inhibition of this phosphorylation. I $\kappa$ B $\alpha$  degradation after TNF- $\alpha$  treatment was not inhibited by NAC or PW, as complete disappearance of the molecule was evident 10 min after the addition of TNF- $\alpha$  alone and in the presence of the antioxidants. In TNF- $\alpha$ -treated endothelial cells, I $\kappa$ B $\alpha$  was resynthesized 40 min after treatment, whereas in PW-treated cells this de novo synthesis was not observed (Fig. 1B). In cells treated with NAC, albeit inhibition of p65 nuclear translocation, I $\kappa$ B $\alpha$  was resynthesized (Fig. 1A).

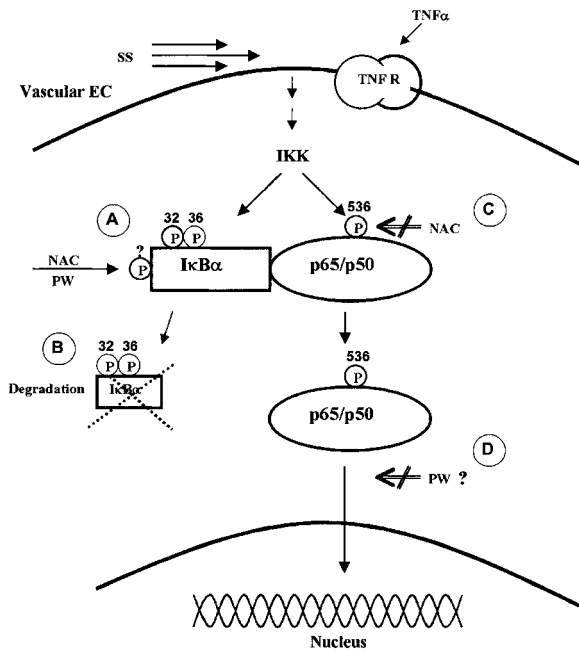
### 3. NAC but not PW inhibit TNF- $\alpha$ -mediated p65 phosphorylation

Phosphorylation of NF- $\kappa$ B was recently suggested as an additional step in the cascade of events leading to NF- $\kappa$ B activation. This phosphorylation is correlated

with the binding of the nuclear NF- $\kappa$ B to the DNA and the formation of nuclear complexes with transcription factor coactivators or repressors. By using an antibody that specifically recognizes Ser536 phosphorylated-p65, we demonstrated that in endothelial cells stimulated with TNF- $\alpha$ , p65 phosphorylation was evident 5 and 10 min after the treatment. Neither NAC nor PW alone had any effect on p65 basal phosphorylation. If added before TNF- $\alpha$ , however, NAC inhibited TNF- $\alpha$ -mediated p65 Ser536 phosphorylation whereas PW had no effect on this phosphorylation (Fig. 1C, D).

### 4. NAC and PW lead to rapid serine phosphorylation of I $\kappa$ B $\alpha$

Preincubation with NAC or PW before the addition of TNF- $\alpha$  (Fig. 2A, B: NAC, 0 min or PW, 0 min) resulted in I $\kappa$ B $\alpha$  serine phosphorylation not observed in control untreated cultures. Nuclear and cytoplasmic fractions were prepared from the cells treated with NAC or PW and I $\kappa$ B $\alpha$  serine phosphorylation, I $\kappa$ B $\alpha$  degradation, and p65 nuclear translo-



**Figure 2.** Novel mechanism for the inhibition of NF- $\kappa$ B by antioxidants in vascular endothelial cells. Treatment of vascular endothelial cells with NAC or PW after stimulation by either cytokines (TNF- $\alpha$ ) or biomechanical forces (shear stress) inhibits NF- $\kappa$ B nuclear translocation and DNA binding. NAC and PW both induce a rapid serine phosphorylation of I $\kappa$ B $\alpha$  on residues other than 32/36, which is not accompanied by inhibition of I $\kappa$ B $\alpha$  degradation or NF- $\kappa$ B nuclear translocation (A). NAC and PW do not inhibit phosphorylation and degradation of I $\kappa$ B $\alpha$  in cells stimulated by TNF- $\alpha$  (B). NAC but not PW inhibits phosphorylation of p65 on serine 536 (C). It remains to be defined how NAC and PW inhibit p65 nuclear translocation in stimulated endothelial cells and whether this inhibition is specific to members of the NF- $\kappa$ B family (D).

cation were tested. Brief treatment of BAEC (10 min) with either NAC or PW resulted in impressive phosphorylation of I $\kappa$ B $\alpha$  on serine residues. Nevertheless, this phosphorylation was accompanied by neither I $\kappa$ B $\alpha$  degradation, nor p65 nuclear translocation. Using an antibody that specifically recognizes serine 32-phosphorylated I $\kappa$ B $\alpha$ , we have shown that treatment of endothelial cells with TNF- $\alpha$  led to a rapid (5 min) Ser32-I $\kappa$ B $\alpha$  phosphorylation, but treatment with NAC or PW alone did not induce this phosphorylation beyond control levels. Treatment of the cells with NAC or PW before the addition of TNF- $\alpha$  did not inhibit Ser32-I $\kappa$ B $\alpha$  phosphorylation.

## CONCLUSIONS AND SIGNIFICANCE

Many vascular diseases result from or accompanied by inflammatory processes, where activation of the transcription factor NF- $\kappa$ B plays a central role by augmenting the expression of inflammatory genes such as cytokines and chemokines, leukocyte-adhe-

sion molecules, growth factors, and matrix-degrading enzymes. Among these diseases is atherosclerosis, where the presence of activated NF- $\kappa$ B in the lesions or regions predisposed for lesion formation was demonstrated. Thus, inhibition of NF- $\kappa$ B activation seems as a preferred target in the development of anti-inflammatory/atherosclerosis drugs. Nevertheless, multiple steps in the NF- $\kappa$ B activation cascade can be marked as targets for this inhibition, where definition of the mechanism through which each drug acts may prove beneficial to achieve synergistic effects. Here we report for the first time that although NAC, PW, and RW were all potent inhibitors of NF- $\kappa$ B activation in vascular EC, each acted through a different and novel mechanism that did not involve I $\kappa$ B $\alpha$  Ser32/36 phosphorylation or stabilization. Whereas NAC inhibited TNF- $\alpha$ -mediated p65 Ser536 phosphorylation, PW had no effect. Phosphorylation of both I $\kappa$ B $\alpha$  and NF- $\kappa$ B is attributed to the IKK complex. The fact that NAC inhibited p65, but not I $\kappa$ B $\alpha$  phosphorylation may point to a differential inhibition of IKK activity by NAC or, alternatively, may suggest the presence of an additional kinase for p65 phosphorylation. A recent study suggests that within the IKK complex, IKK $\alpha$  is solely responsible for p65 phosphorylation whereas IKK $\beta$  is capable of phosphorylating both I $\kappa$ B and p65. NAC and PW also differed in their effect on late de novo synthesis of I $\kappa$ B $\alpha$  in TNF- $\alpha$ -treated EC. Where this de novo synthesis occurred in cells treated with NAC and TNF- $\alpha$ , it was not observed in cells treated with TNF- $\alpha$  and PW (Fig. 1A*b*, B*b*). As I $\kappa$ B $\alpha$  transcription is dependent on NF- $\kappa$ B, it is not surprising that PW, which inhibits NF- $\kappa$ B activation, also inhibits I $\kappa$ B $\alpha$  resynthesis. It is not clear why I $\kappa$ B $\alpha$  de novo synthesis is unaffected in NAC-treated cells unless alternative transcription factors promote this synthesis. Nor is it clear what step in NF- $\kappa$ B activation cascade is inhibited by PW, as neither I $\kappa$ B $\alpha$  phosphorylation and degradation nor p65 phosphorylation is inhibited by this antioxidant. Three additional antioxidants—gallic acid, caffeic acid phenethyl ester, and herbimycin A—inhibit NF- $\kappa$ B activation through an unknown mechanism which does not involve the inhibition of I $\kappa$ B $\alpha$  phosphorylation or degradation. Gallic acid has been shown to be the major compound in pomegranate juice.

Development of novel NF- $\kappa$ B inhibitory drugs is important in prevention and treatment of cardiovascular diseases. Natural antioxidants of food substances (such as red wine and pomegranate wine) have a great advantage over existing drugs as they are nontoxic and inexpensive. Their potency as NF- $\kappa$ B inhibitors in arterial endothelial cells in culture and the novel mechanisms through which they inhibit NF- $\kappa$ B activation pathway provide a strong rationale for further studying these reagents in vitro and in vivo. **FJ**