Effect of Apple Extracts on NF-κB Activation in Human Umbilical Vein Endothelial Cells

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The mechanisms by which foods, such as fruit, are able to reduce the risk of chronic disease are still unclear. Several fruit products, including apples and apple juice, that are flavonoid-rich are reported to increase antioxidant levels in human subjects. This is supported by the finding from our previous studies that the chronic consumption of apple juice by human subjects reduced *ex vivo* low-density lipoprotein (LDL) oxidation; we hypothesized that this was due to the flavonoid in the apple juice, which, as we reported earlier, reduced *in vitro* LDL oxidation. To further explore whether the mixture of flavonoids and other phytochemicals in apples are biologically relevant antioxidants, we tested the effects of this flavonoid-rich apple extract (AE) on oxidant-related pathways in a model of the endothelium: human umbilical vascular endothelial cells (HUVECs). The effects of AE on oxidant-responsive (i.e., tumor necrosis factor [TNF]-α–induced) nuclear factor (NF)-κB signaling in cell culture were assessed in transfected HUVECs by using a construct that expressed luciferase under the control of NF-κB. Incubation of HUVEC for 24 hrs with up to 10 m*M* (as gallic acid equivalents) of AE demonstrated no cytotoxicity, as determined by lactate dehydrogenase release, caspase 3 activation, and apoptosis marker–based FACS analysis. AE after a 24-hr incubation period at either 200 or 2000 n*M* showed a complex pattern of decreased basal and TNF-α–stimulated NF-κB signaling (63% maximal decrease) as assessed by luciferase activity in the transfected HUVECs, as well as by reduced levels of IκBα protein phosphorylation detected by Western blot analysis. We suggest that AE downregulates NF-κB signaling and that this is indicative of an antioxidant effect of the flavonoids present in AE. Exp Biol Med 231:594–598, 2006

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Introduction

Despite an enormous interest in the relationship between plant polyphenolic compounds and health, the exact contributions of these compounds to human health and the mechanism(s) by which they act are unclear (1, 2). One frequently proposed mechanism is that some classes of phytochemicals act in part *via* their ability to augment the body's antioxidant defense system. This hypothesis has been widely cited in support of the concept that diets rich in flavonoids can reduce the risk for cardiovascular disease. Although a large body of literature supports this idea, the collective evidence for the hypothesis is viewed as inconclusive. We previously reported that the chronic consumption of apple juice can reduce the oxidation of low-density lipoprotein (LDL) *ex vivo* (3) and that this effect was due to the flavonoid content in the juice. We suggested that apple juice could be cardioprotective, because oxidized LDL is thought to be a contributory factor to the development and progression of cardiovascular disease (CVD). Recently, however, the role of apple-derived flavonoids as antioxidants has been questioned, and it has been alternatively suggested that apple juice consumption increases plasma antioxidant capacity *in vivo* as a result of uric acid production secondary to fructose metabolism (4).

Although this hypothesis clearly merits more investigation, particularly given the recent reports of the negative effects of urate (5) in contrast to the health benefits attributed to fruit consumption (6), there are additional findings that suggest that plant flavonoids can contribute to oxidant defense. For example, *in vivo* effects of almond skin–derived flavonoids showed that these compounds exhibit a synergy with tocopherol in their ability to protect LDL from *ex vivo* oxidation in animals fed almond skin (7). This synergy may also occur in the case of apples: Filipe *et*

al. (8) have shown that urate and flavonoids, such as quercetin, demonstrate a synergy in terms of their collective effects on lipoprotein oxidation. In the present study, we investigated the hypothesis that the phytochemicals present in apples can downregulate the nuclear factor (NF)-κB signal transduction pathway, which responds to oxidative signals. We tested the ability of AE to alter the response to tumor necrosis factor (TNF)-α in human umbilical vascular endothelial cells (HUVECs), which are a model system widely used to identify the effects of and targets for deleterious cardiovascular risk factors, such as hypercholesterolemia and oxidative stress on endothelial cells (9, 10). TNF-α was selected as a stressor because it has been shown that TNF-α–induced cell responses, particularly in endothelial cells, are mediated through its ability to promote intracellular reactive oxygen species (ROS) formation (11, 12). The activation status of NF-κB was used as a means to assess HUVEC responses to oxidants (13) because it has been linked to a wide range of diseases (14). For example, uncontrolled or chronic NF-κB activation is a hallmark of cancer and chronic inflammatory diseases (15). The aims of the current study are to examine whether a flavonoid-rich AE has positive antioxidant effects in a system that does not involve the potential cofounder of fructose-induced urate production. We did this by probing AE effects on the NF-κB signaling system, which is known to respond to oxidative stress.

Materials and Methods

Apple Extract. Fresh apples (premium-sized Fuji, Golden Delicious, Red Delicious, and Granny Smith apples from Washington State that were purchased from a local grocery) were washed with tap water and air dried, and an extract was prepared as previously described by Pearson *et al.* (16); these researchers demonstrated that this extract contains a wide range of apple-derived phytochemicals.

Determination of the Total Phenol Level. The total level of phenol was determined using gallic acid as a standard, and levels were expressed as gallic acid equivalents (GAEs) (17).

Determination of NF-κB Activity. To test the antioxidant activity of apple extracts *in vitro*, HUVEC cultures were pretreated with varying amounts of AE for 24 hrs before the addition of TNF-α, using a midrange of AE doses assessed for cytotoxicity. HUVECs were transfected using Lipofectamide (20%–30% efficiency) and allowed to recover for 24 hrs. AE was then added and incubated for an additional 24 hrs, after which cells were incubated for various periods with 100 n*M* TNF-α. The effect of the AE pretreatment on TNF-α–induced responses was assessed by using a NF-κB-driven luciferase-producing construct controlled by a synthetic promoter that contains direct repeats of the transcription recognition sequences for NF-κB binding sites (PathDetect NF-kB Cis-Reporting System; Stratagene, Cedar Creek, TX). In addition, a positive-control plasmid (pFC-MEKK) was used that expressed a kinase from the constitutive CMV promoter that is ultimately responsible for activating transcription of the luciferase from the enhancer element–TATA box region for NF-κB.

Equal numbers of HUVECs in triplicate were transfected with constructs and allowed to grow overnight. The cells were then incubated for 24 hrs with AE, after which 100 n*M* TNF-α was added for a fixed period. The cells were then harvested and lysed using the cell lysis buffer provided with the luciferase assay kit (Luciferase Assay Kit; Stratagene). The level of luciferase activity was assessed by counting light emission in a luminometer (Luciferase Assay Kit).

The results of testing AE's effects on luciferase expression driven by NF-κB activity were assessed using the luciferase levels in experimental groups divided by the unstimulated transfected control cells. Statistical analysis was performed by comparing the response from various different treatment groups.

Determination of the IκBα Level. The effect of AE on the IκBα pathway was determined by using antibodies directed against phosphorylated IκBα (P-IκBα) to assess the level of phosphorylated protein in Western blots of HUVEC proteins. Western blots were performed using protein extracts of HUVECs incubated with no addition (control), AE, AE and TNF-α, or TNF-α alone for the indicated times described by Valacchi *et al.* (18). Gels were loaded with equal amounts of protein as assessed by both protein assay and by immunostaining of the blots for detection of tubulin.

Cytotoxicity Measurements. The possible cytotoxicity of the AE was assessed by measuring lactate dehydrogenase (LDH) release. LDH is a stable cytosolic enzyme that is released upon cell lysis, and the amount of released LDH in culture supernatants can be measured with a coupled enzymatic assay that results in the conversion of a tetrazolium salt (INT) into a red formazan product (19).

Two additional measurements of the effects of AE on cell health were performed. One assayed the activity of caspase 3, an important caspase involved in apoptosis. This was assessed by collecting adherent and floating HUVECs after treatment with AE (20, 200, or 2000 n*M*) for 24 hrs. Cells were washed twice with phosphate buffer solution and then lysed in 100 μl of lysis buffer (50 m*M* HEPES, 5 m*M* CHAPS, and 5 m*M* DTT) in an ice-bath for 20 mins and centrifuged (15,000 *g* for 15 mins) at 4°C. The proteins present in the supernatants were quantified using Coomassie Protein Assay (BioRad, Hercules, CA), diluted to equal concentration, and then incubated with 50 μ*M* of caspase 3 (Ac-DMQD-AMC) substrate (Alexis Inc., Lausen, Switzerland) in an assay buffer (20 m*M* HEPES, 2.5 m*M* CHAPS, 5 m*M* DTT, and 2 m*M* EDTA) at 37°C for 4 hrs. The level of caspase 3 was assessed by measuring the release of a fluorescent product with a microplate reader (FluoroCount [360-nm excitation and 460-nm emission]; Packard Instrument Co., Meriden, CT).

The final assessment used fluorescence activated cell

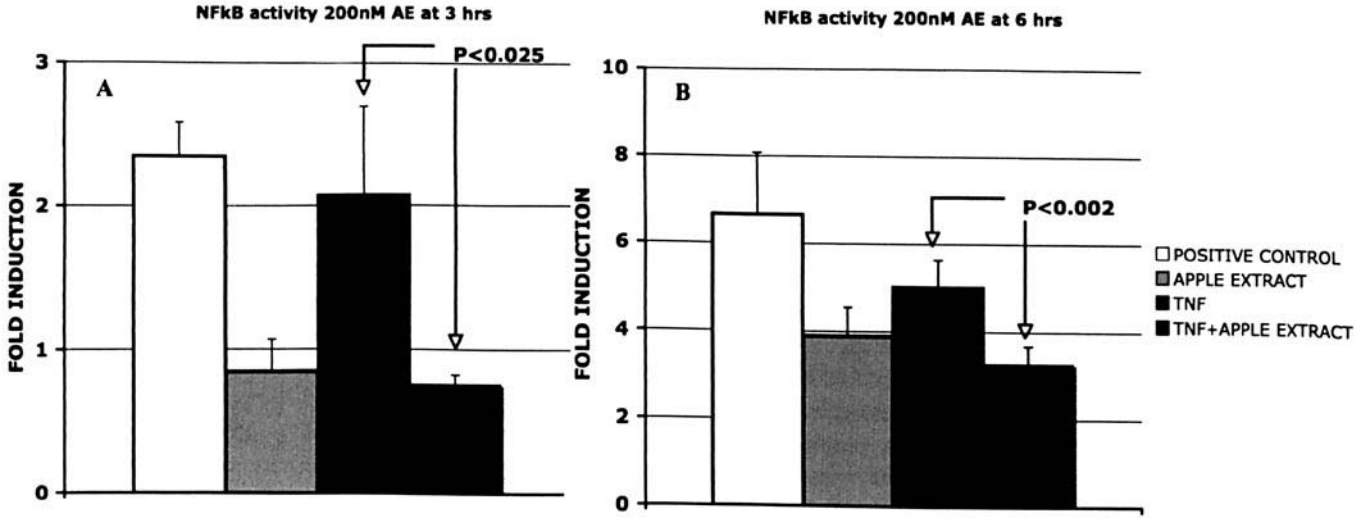

Figure 1. (A) The effect of 200 n*M* apple extract (AE) on TNF-α–stimulated NF-κB–driven luciferase expression in HUVECs in culture after 3 hrs of TNF-α exposure. (B) The effect of 200 n*M* AE on TNF-α–stimulated NF-κB–driven luciferase expression in HUVECs in culture after 6 hrs of TNF-α exposure. HUVECs were transfected and allowed to recover for 24 hrs, after which 200 n*M* AE was added, and the mixture was incubated for an additional 24 hrs. Cells were then incubated with 100 n*M* TNF-α for either 3 hrs or 6 hrs and harvested. Luciferase activity was assayed, and the results are presented as the level of luciferase in experimental groups divided by the level in unstimulated control transfected cells ± SEM. A plasmid (pFC-MEKK) that maximally induces luciferase was used as a positive control to assess transfection.

sorter (FACS)-based analysis to examine the distribution of two different cell status markers (i.e., apoptosis and viability). Staining of HUVECs was conducted using fluorescein isothiocyanate-conjugated annexin V (Invitrogen Corp., Carlsbad, CA), an apoptosis marker, and propidium iodide (a vital stain) after incubation with AE for 24 hrs, and results were analyzed using flow cytometry (FACS Star 488-nm argon laser for excitation; BD, Franklin Lakes, NJ). Ten thousand cells with scatter characteristics of viable cells were collected for each sample. The cell population for analysis was gated using forward versus side-scatter parameters. Flow cytometric data were analyzed using Cell Quest Pro software (BD).

Statistical analysis. Data were analyzed by means of ANOVA in Excel (Microsoft, Redmond, WA), with $P < 0.05$ considered to be statistically significant.

Results

Cytotoxicity testing by means of analysis of LDH release, caspase 3 activity, or FACs of AE effects on HUVEC populations demonstrated no cytotoxic effects of AE up to the highest concentration tested: 10 m*M* GAE (data not shown).

Assays done at the 1- or 2-hr incubation time point with TNF-α after preincubation with either 20 or 200 n*M* AE resulted in non–statistically significant reductions in TNF-α–stimulated luciferase activity (data not shown). At the longer TNF-α exposure time points, 200 n*M* AE showed statistically significant decreases in TNF-α–stimulated luciferase activity, compared with TNF-α alone (63% vs. 35%), at 3 and 6 hrs after TNF-α stimulation, respectively (Fig. 1A, B). Preincubation with 2000 n*M* AE resulted in a statistically significant (i.e., approximately 50%) reduction in TNF-α–stimulated activity when assessed 6 hrs after TNF-α addition (Fig. 2).

Western blot analysis of P-IκBα levels, as shown in Figure 3, indicated that 2- and 3-hr exposure of HUVECs to 20, 200, and 2000 n*M* AE resulted in reduced P-IκBα expression.

Discussion

HUVECs did not show any sign of cytotoxicity after exposure to AE, even at extremely high doses (10 m*M*).

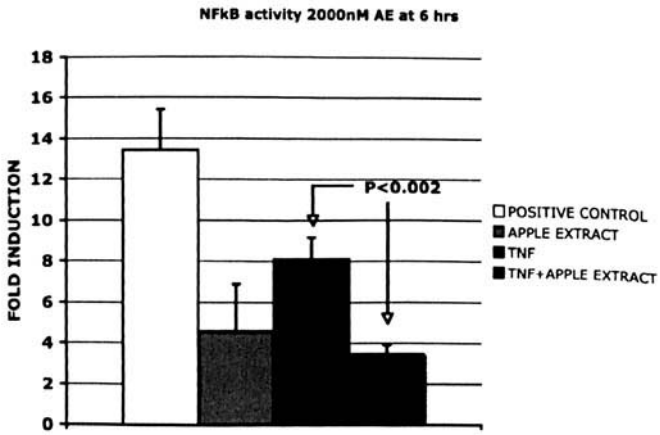

Figure 2. The effect of 2000 n*M* apple extract (AE) on TNF-α–stimulated NF-κB–driven luciferase expression in HUVECs in culture after 6 hrs of TNF-α exposure. HUVECs were transfected and allowed to recover for 24 hrs. A total of 2000 n*M* AE was added, and the mixture was incubated for an additional 24 hrs, after which cells were incubated with 100 n*M* TNF-α for 6 hrs and harvested. Luciferase activity was assayed, and the results are presented as the level of luciferase in experimental groups divided by the level in unstimulated control transfected cells ± SEM. A plasmid (pFC-MEKK) that maximally induces luciferase was used as a positive control to assess transfection.

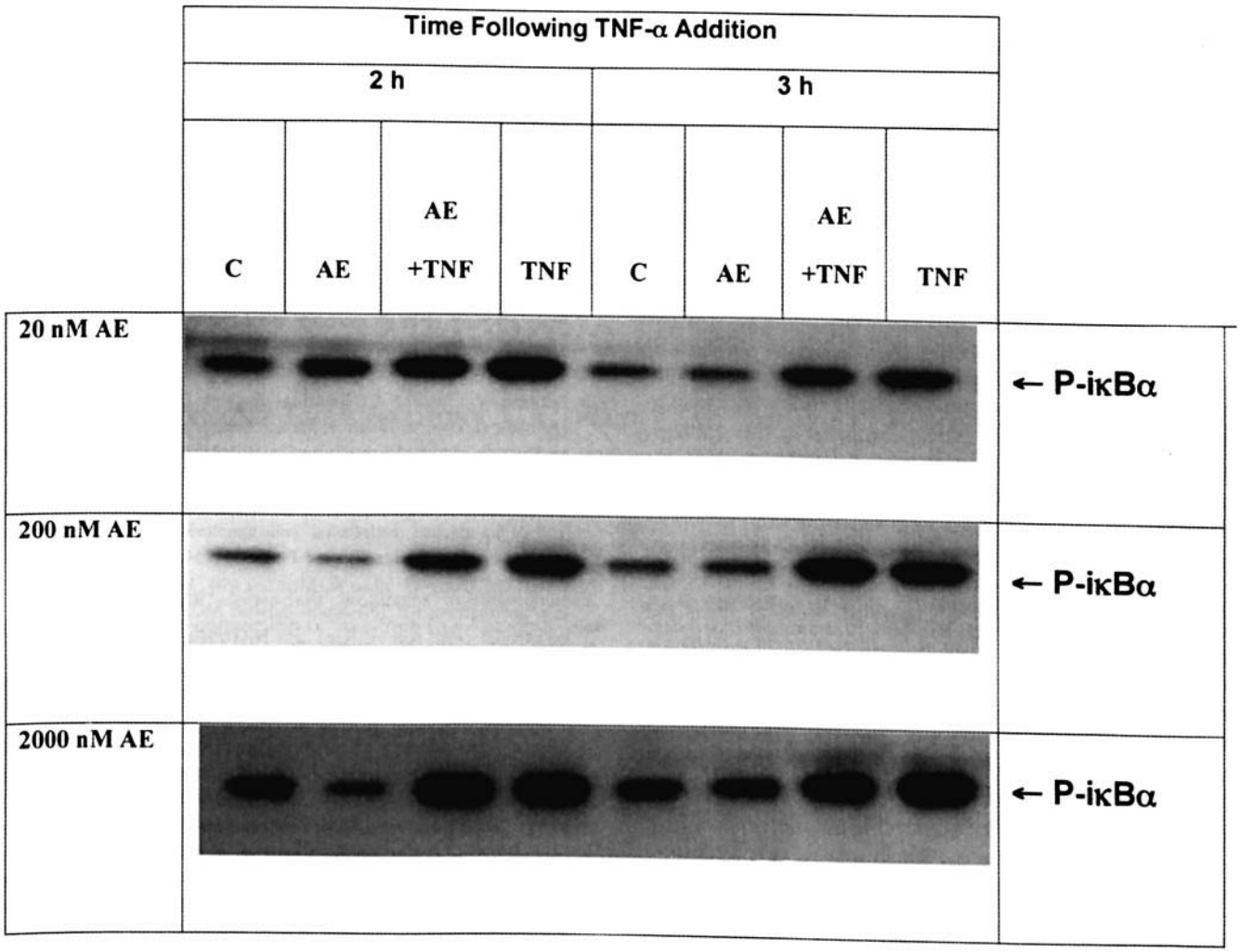

Figure 3. The effect of apple extract (AE) on the phosphorylation of IκBα, as assessed by Western blot analysis with antibodies directed at IκBα to determine the level of the phosphorylated IκBα protein in HUVEC protein extracts after separation by gel electrophoresis. Representative Western blot results are presented for HUVEC proteins extracted after incubation with no additions (control), AE, AE and TNFα, or TNFα alone. A representative blot is shown.

Data from this study indicate that HUVECs exhibited no overt cytotoxicity when exposed to AE over a 500-fold concentration range in the following three assay systems: absence of LDH release, activation of caspase 3, and activation of apoptosis markers.

Apple extract produced an increasing inhibition of the TNF-α signal *via* NF-κB in HUVECs; however, the inhibitory effects only reached statistical significance at higher AE concentrations (200–2000 n*M*) and at longer TNF-α incubation periods (3–6 hrs). The reductions in NF-κB signaling appear to be a direct result of the activity of AE, because HUVECs are thought to be relatively resistant to TNF-α–induced apoptosis (20), thus suggesting that induction of apoptosis is an unlikely reason for the decrease in luciferase activity. Western blot results are congruent with the luciferase data and suggest that AE effects are mediated, in part, by AE inhibition of the required IκBα phosphorylation step in the NF-κB activation process. This prevents the disaggregation and nuclear localization of NF-κB and, in doing so, inhibits the effects of NF-κB. Wheeler *et al.* (21) have documented a similar inhibitory effect of tea polyphenolics on the IL-1β–dependent IκBα phosphorylation step in epithelial cells. Interestingly, in the current study, both the luciferase-based assay and probing for IκBα phosphorylation at the earliest time points and highest levels of AE revealed an apparent increase in NF-κB activity. The significance of this is unclear but suggests that there may be some effect of AE on other NF-κB–related systems that are not associated with the one probed by TNF-α. This would be contrary to the consistent inhibition of HUVEC NF-κB signaling by AE, as observed here, in that AE would be expected to accentuate TNF-α–induced NF-κB signaling activity over and above that observed for TNF-α alone. We observed that AE affected the NF-κB–driven luciferase signal and that AE exhibited both time- and concentration-dependent effects on both basal and NF-κB–driven TNF-α–evoked response. This suggests that there are likely multiple mechanisms for AE inhibition of TNF-α–driven NF-κB

activity. We should note that HUVECs do not produce urate from fructose; the urate from fructose is primarily a hepatocyte-based response. Thus, although the production of urate *in vivo* is a confounder when assessing the antioxidant activity of apple consumption, it is not a confounder in the current study (5).

The results of the current study demonstrate marked differences between the effects of AE flavonoids and those reported for grape seed proanthocyanidin extracts in HUVECs, in which no effect on NF-κB–related processes was observed (22). The reasons for the differences between these two extracts likely resides in the different species of phytochemicals present in the extracts. A recent observation by Liu *et al.* (23), documenting the ability of apple extract to prevent mammary cancer, merits comment in this context. One aspect of their finding that is similar to findings of our study is that apple polyphenolics have discernible biological effects that can be associated with health effects in whole animal studies. Liu *et al.* (23) argue that their observations imply that a multiplicity of chemical entities found in AE are involved in a "total" AE activity. Results from the current study and those of Liu *et al.* (23) suggest a need for future experiments that more fully identify and characterize pathways responsible for the biologic activity of apples. The results presented here and those of Liu *et al.* (23) imply that diseases characterized by NF-κB activation (such as cancer) may represent an important area in which to investigate the potential health benefits of apple consumption. Nakanishi and Toi (24) have recently suggested that NF-κB inhibitors may function as sensitizers to anticancer drugs, and in light of our observations that AE can inhibit NF-κB signaling, flavonoid-rich foods—such as apples—may be useful as possible adjuncts to chemotherapy. Because apoptosis is a part of the body's cancer surveillance system, the ability of AE to inhibit NF-κB pathways may assist in the removal of potentially cancerous cells targeted for elimination. Thus, flavonoid-rich foods (e.g., apples) might also function in a manner that prevents cancer (25).

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