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Dietary (−)-epicatechin affects NF-κB activation and NADPH oxidases in the kidney cortex of high-fructose-fed rats

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Inflammation involves the activation of redox-sensitive transcription factors, *e.g.*, nuclear factor κB (NF-κB). Administration of (−)-epicatechin to high-fructose-fed rats prevented NF-κB activation and up-regulation of the NADPH oxidase 4 (NOX4) in the kidney cortex. These results add mechanistic insights into the action of (−)-epicatechin diminishing inflammatory responses.

Introduction

A chronic low-grade inflammatory state is associated with a wide range of chronic conditions such as metabolic syndrome, non-alcoholic fatty liver disease, type 2 diabetes mellitus and cardiovascular disease. The inflammatory process is initiated by the up-regulation of genes encoding for diverse mediators, such as cytokines and chemokines, through the activation of different transcription factors including nuclear factor κB (NF-κB).[1]

NF-κB can be activated by different additional and intracellular stimuli.[2] NF-κB is a heterodimer composed of members of the Rel/NF-κB family of proteins (*e.g.*, p50 and p65) and resides in the cytosol in an inactive form bound to the inhibitory protein κBα (IκBα). During NF-κB activation, IκBα kinases (IKKα/β) phosphorylate IκBα leading to its subsequent ubiquitination and degradation by the proteasome. The released NF-κB is susceptible to phosphorylation in p65 which allows the translocation to the nucleus, where it binds to κB sites in DNA and regulates the transcription of proinflammatory mediators such as interleukin 6, TNFα, and inducible nitric oxide synthase (iNOS).[3] The role of toll-like receptors (TLR) in NF-κB activation is well characterized. Mammalian TLRs are sensors of pathogen-associated molecules such as bacterial lipopolysaccharides, lipopeptides and flagellins as well as of endogenous ligands such as diverse damage-associated molecules. TLR-4 recognizes lipopolysaccharides from Gram-negative bacteria and endogenous molecules such as heat shock protein 60, fibronectin and fibrinogen, leading to the activation of the NF-κB signalling pathway.[2]

The family of NADPH oxidases (NOX) consists of several superoxide anion- or H_2O_2-producing enzymes. In the rat kidney, NOX1, NOX2 and NOX4 are expressed in mesangial cells, vascular smooth muscle cells, endothelial cells, podocytes, tubular epithelial cells and interstitial fibroblasts. NOX2 consists of a membrane-bound catalytic subunit ($gp91^{phox}$) and regulatory/activating cytosolic subunits, *e.g.*, $p47^{phox}$ and $p67^{phox}$.[4] The isoform NOX1 has a membrane-bound catalytic subunit with the same name (NOX1) and specific cytosolic regulatory/activating subunits, *e.g.*, NOX1 organizer (NOXO1). Electrons from NADPH are transferred to molecular oxygen, generating the superoxide anion as a product of NOX1 and NOX2 activity. NOX4 activation does not require the recruitment of cytosolic subunits and generates H_2O_2 rather than the superoxide anion.[5]

Flavanol (−)-epicatechin, which is present in many plants consumed by humans, has been proposed as a modulator of NOX-dependent oxidant production *in vivo* in many tissues subjected to stress conditions.[6,7] The mechanisms behind such effects imply the inhibition of the overexpression of different NOX subunits.[8–16] Moreover, *in vivo* anti-inflammatory effects have been reported for (−)-epicatechin through downregulation of the NF-κB activation pathway in fructose overload and other stress models.[8,11,14–16] In this study, high fructose feeding triggered NF-κB activation in the renal cortex of rats. This was associated with increase in NOXO1 and NOX4 expression levels without changes in TLR-4 expression. Dietary (−)-epicatechin could prevent NF-κB activation at multiple steps in association with a decrease in NOX4 expression.

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Materials and methods

Materials

Primary antibodies for TLR-4 (#30002), NOX4 (#21860) and β-actin (#47778) and secondary antibodies rabbit anti-goat IgG-HRP (#2768), mouse anti-rabbit IgG-HRP (#2357), and goat anti-mouse IgG-HRP (#2005) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The primary and secondary antibodies for IκB kinase α (IKKα) (#11930), p-IKKα/β (Ser176/180) (#2697), inhibitor of κBα (IκBα) (#4814), p-IκBα (Ser32) (#2859), NF-κB p65 (#8242) and p-NF-κB p65 (Ser536) (#3033) were obtained from Cell Signaling Technology (Boston, MA, USA). Primary antibodies for NOX1 (ab46545) and NOXO1 (ab131205) were purchased from Abcam Inc. (Cambridge, MA, USA). (−)-Epicatechin was obtained from Sigma Chemical Co. (St Louis, MO, USA), and fructose (purity ≥99.5%) was obtained from Droguería Saporiti (Buenos Aires, Argentina). Commercial rat chow was purchased from Gepsa-Feeds (Buenos Aires, Argentina).

Animals, diets and experimental design

All procedures were in agreement with the standards for the care of laboratory animals outlined in the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH Pub. No. 85-23, revised 1996) and were approved by the Secretary of Science and Technology of the School of Medicine, National University of Cuyo. Male Sprague Dawley rats were housed under the conditions of controlled temperature (21–25 °C) and humidity with a 12 h light/dark cycle. Rats weighing 130 ± 20 g were randomly divided into the following groups (8 rats per group): control (C), receiving tap water *ad libitum* and the standard rat chow diet; fructose (F), receiving 10% fructose (w/v) in the drinking water and the standard rat chow diet; and fructose-(−)-epicatechin (FE), receiving 10% fructose (w/v) in the drinking water and the standard rat chow diet with (−)-epicatechin added to reach 20 mg per kg of body weight per d.[14] The (−)-epicatechin provided to the rats is equivalent to 200 mg d^{-1} for a 70 kg human (considering a factor of 7 to convert rat and human doses),[17] an amount that is attainable through optimizing fruit and vegetable intake and/or pharmacological strategies.[9,18] After 8 weeks on the respective treatments, the rats were weighed and anesthetized with ketamine (50 mg per kg of body weight) and acepromazine (1 mg per kg of body weight). The kidneys were excised, and the kidney cortex was immediately isolated and processed for western blot analysis.

Western blot analysis

A portion of the renal cortex was homogenized in lysis buffer (150 mM NaCl, 50 mM Trizma-HCl, 1% (v/v) NP-40, pH: 8.0) in the presence of protease and phosphatase inhibitors and centrifuged at 600*g* for 20 min. The supernatant was collected and used as the total homogenate. Protein content was measured by the Lowry method. The homogenates were added with 2× solution of sample buffer (62.5 mM Tris-HCl, pH: 6.8 containing 2% (w/v) SDS, 25% (w/v) glycerol, 5% (v/v) β-mercaptoethanol, and 0.01% (w/v) bromophenol blue) and heated at 95 °C for 2 min. Sample aliquots containing 30 μg of protein were subjected to reducing 10% (w/v) polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes. Colored molecular weight standards (GE Healthcare, Piscataway, NJ, USA) were run simultaneously. Membranes were blotted for 2 h in 5% (w/v) nonfat milk and incubated overnight in the presence of the corresponding primary antibodies (1 : 1000 dilution in PBS). After subsequent incubation for 90 min at room temperature in the presence of the corresponding HRP-conjugated secondary antibody (1 : 5000 dilution in PBS), the complexes were visualized by chemiluminescence. Films were scanned, and densitometric analysis was performed using Image J (National Institute of Health, Bethesda, Maryland, USA). The protein bands were normalized to the β-actin content.

Statistical analysis

Results are shown as mean ± SEM. Data were analyzed by one-way analysis of variance (ANOVA) using StatView 5.0 (SAS Institute, Cary, NC, USA), and Fisher's significance difference test was used to examine the differences between group means. A value of $p < 0.05$ is considered statistically significant.

Results and discussion

We have previously shown that (−)-epicatechin attenuated the renal pro-inflammatory state induced by the consumption of high-fructose diet in rats. This was associated with the attenuation of NOX2 activation.[8] To extend these results, in the present study, we evaluated the effects of (−)-epicatechin on the activation of NF-κB and on NOX expression. The obtained results indicate that by preventing the activation of select NOXs, (−)-epicatechin prevents the activation of NF-κB in the kidney cortex.

In a preceding paper using the same set of rats, both high-fructose feeding and (−)-epicatechin modified several metabolic and kidney function parameters. Of importance for the present results, kidney/body weight and serum creatinine were not modified by the diets, but increased proteinuria (71%, F with respect to C) associated with fructose consumption was not observed in FE rats. These results indicate that in F rats, there was incipient renal damage that was not enough in extent and/or time to affect most metabolic parameters and damage was prevented by (−)-epicatechin administration.[8]

TLR-4 is key in mediating NF-κB activation initiated extracellularly.[19] In this study, no changes were found in TLR-4 expression in the kidney cortex as a result of both high-fructose feeding and (−)-epicatechin administration (Fig. 1A). These results suggest that most of the observed changes are not dictated by the basal expression of TLR-4 and can possibly be events initiated by intracellular agents such as oxidants. The observed unmodified basal levels of TLR-4 are in contrast with other previously reported results showing an over-

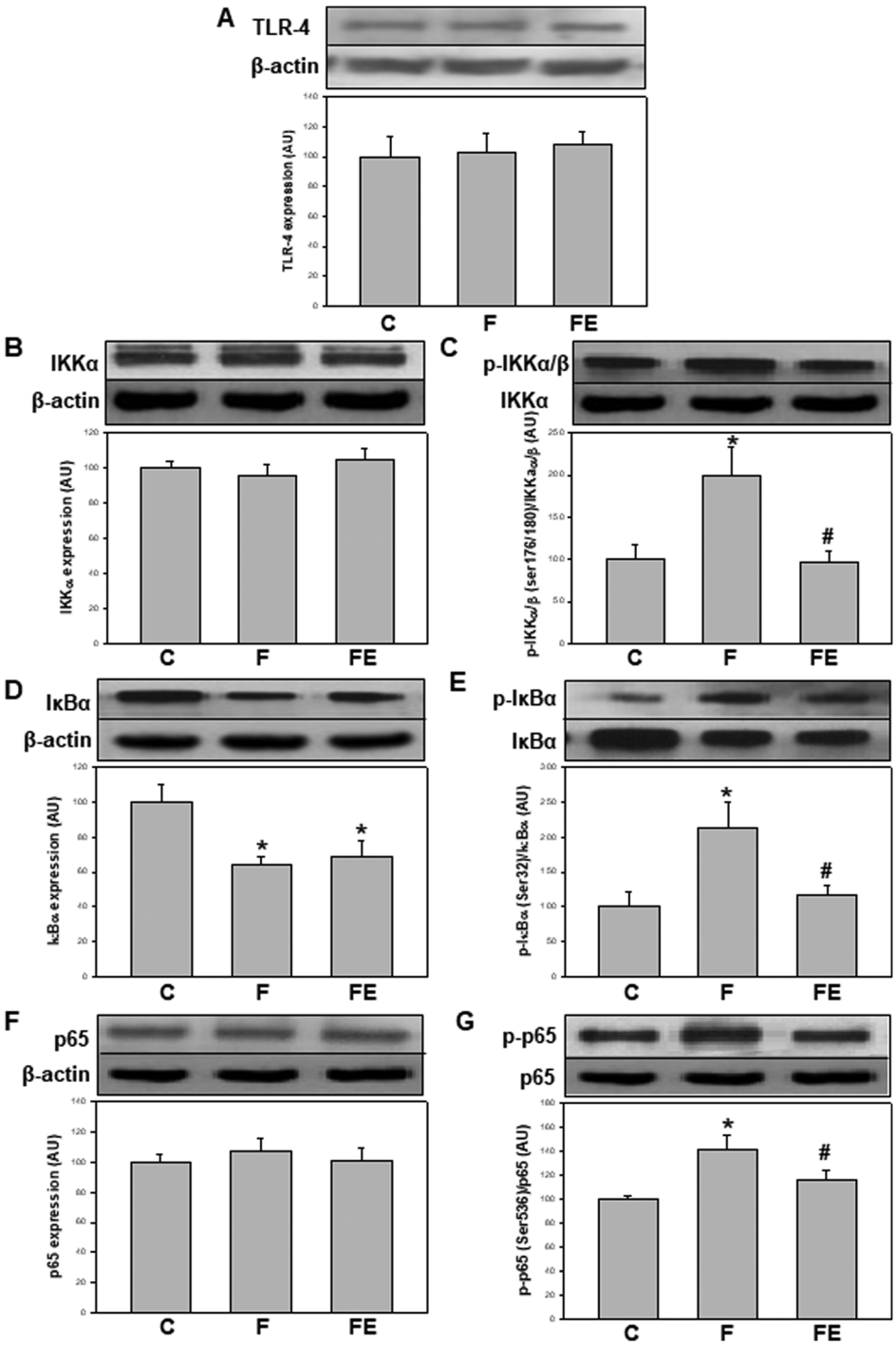

Fig. 1 Effects of dietary (−)-epicatechin on TLR-4 expression and NF-κB signaling pathway in the renal cortex of high-fructose-fed rats. TLR-4 expression (A), IKKα content (B), IKKα/β phosphorylation at Ser(176/180) (C), IκB content (D), IκBα phosphorylation at Ser(32) (E), NF-κB p65 content (F) and NF-κB p65 phosphorylation at Ser(536) (G) determined by western blot in homogenates of the renal cortex from C: control, F: fructose, and FE: fructose-(−)-epicatechin groups. Values are expressed as mean ± SEM; n = 8 rats per group. *$p < 0.05$ respect to C, $^{\#}p < 0.05$ respect to F.

expression of TLR-4 in the kidney of high-fructose-fed[20–22] or LPS-treated[11,23] rodents. It is important to note that in LPS-treated rats, TLR-4 was overexpressed, and (−)-epicatechin was active to prevent such overexpression and the associated activation of NF-κB.[11]

NF-κB activation occurs through several regulatory events. IKKα content was similar in the three experimental groups (Fig. 1B). Ikkα/β phosphorylation at Ser(176/180) (p-IKKα/β) was significantly higher in the F group compared to that in the C and FE groups (+99% and +108%, respectively) (Fig. 1C). The

total content of IκBα, which is degraded during NF-κB activation, was significantly lower in the F and FE groups compared to that in the C group (−37% and −31%, respectively) (Fig. 1D). The levels of IκBα phosphorylation at Ser(32) (p-IκBα) were significantly higher in the F group compared to those in the C and FE groups (+113% and +80%, respectively) (Fig. 1E). NF-κB p65 content showed no changes among the experimental groups (Fig. 1F), but the activating phosphorylation at Ser(536) was significantly higher in the F group compared to that in the C and FE groups (+41% and +22% respectively) (Fig. 1G). These results indicate that (−)-epicatechin inhibited the phosphorylation steps that control the activation of the NF-κB pathway induced by high fructose feeding. Similar modulatory effects of (−)-epicatechin on NF-κB activation were observed in the kidney of LPS-treated rats.[11] However, in that experimental setting, IκBα protein levels were returned to control values by (−)-epicatechin treatment, whereas in the present experiments, (−)-epicatechin had no effect. Such discrepancies between total IκBα protein content and other parameters of NF-κB activation could be ascribed to the complex turnover of IκBα associated with different kinetics of synthesis and degradation of free and NF-κB-bound IκBα.[24]

Oxidant levels in cells are closely associated with NF-κB activation.[25] Early reports have demonstrated that the activation of NF-κB is associated with cellular oxidative stress[26–28] and the expressions of some NOX subunits are regulated by NF-κB.[29,30] Then, a cycle involving NOX-derived oxidants and activation of NF-κB has been proposed and supported by extensive experimental evidence.[31–33] However, the initiating event in this cycle, *i.e.*, the NF-κB pathway activation or NOX activation is highly dependent on challenging conditions and physiological response.[25]

In terms of the regulation of both, oxidant production and NOX activities, we previously showed that the consumption of high-fructose diets increases superoxide anion production and the expression of NOX2 subunits $p47^{phox}$ and $gp91^{phox}$ in rat kidneys. The administration of (−)-epicatechin counteracted the increments in superoxide anion production and only the expression of $p47^{phox}$ in the renal cortex.[8] Here, we investigated the regulation of other NOX isoforms following high fructose consumption and (−)-epicatechin administration. NOX1 expression was similar in the three experimental groups (Fig. 2A), but NOXO1 protein levels were 86% higher in F and FE groups compared to that for the C group ($p < 0.05$) (Fig. 2B). These results suggest greater NOX1 activity mediated by higher NOXO1 expression in high-fructose-fed rats, an effect that was not modified by (−)-epicatechin administration. The absence of the effect of (−)-epicatechin on the activation of NOX-1, especially on NOXO1, can sustain increased superoxide anion production that is responsible for the remaining/

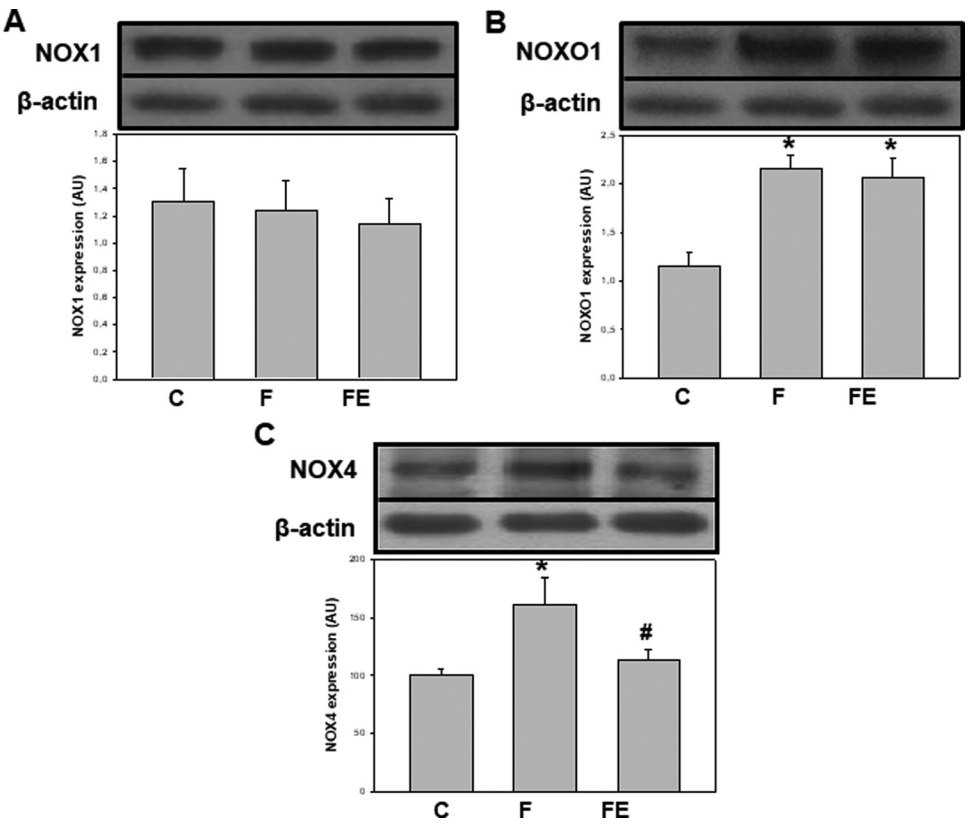

Fig. 2 Effects of dietary (−)-epicatechin on NOX1, NOXO1, and NOX4 expressions in the renal cortex of high-fructose-fed rats. Protein expression levels were measured by western blot in homogenates of the renal cortex from C: control, F: fructose; and FE: fructose-(−)-epicatechin groups. Values are expressed as mean ± SEM; $n = 8$ rats per group. *$p < 0.05$ respect to C, #$p < 0.05$ respect to F.

residual effects of the high-fructose diet on some renal parameters, for which alterations were not completely prevented by (−)-epicatechin. NOX4 expressions were 61% and 46% higher in the F group compared to that for C and FE groups ($p < 0.05$) (Fig. 2C), indicating increased intracellular production of H_2O_2 after high fructose consumption. This effect was prevented by (−)-epicatechin, as it was previously observed in the aorta, liver, and adipose tissue of high-fructose-fed rats.[9,14] This regulation of NOX4 by (−)-epicatechin can be of relevance because NOX4 activity is dictated solely by the expression of this protein, which is a direct manner of controlling oxidant production.

By integrating the present experiments with previous observations,[32–36] we can conclude that renal NOXs responded differentially to a high-fructose diet and to (−)-epicatechin administration. As summarized in Table 1, excessive fructose in the diet was mostly associated with higher NOX activation that followed different patterns: (i) higher expression of NOX2 activating and catalytic subunits, (ii) higher expression of the NOX1 activating subunit, and (iii) higher expression of the NOX4 catalytic subunit. These differences could reflect specific activation mechanisms for each NOX isoform. (−)-Epicatechin supplementation led to changes only in NOX2 and NOX4 that could decrease enzymatic activation by avoiding the overexpression of the organizer subunit of NOX2 and that of the catalytic subunit of NOX4.

Regarding the sequence of NF-κB and NOXs activations, our results suggest that (−)-epicatechin regulates the NF-κB signaling pathway through the modulation of NOX2 and NOX4. Then, the production of superoxide anion by NOX2 and/or the production of H_2O_2 by NOX4 can define the redox regulation of NF-κB activation probably initiated by the phosphorylation and stabilization of IKKα. In this regard, it was demonstrated that H_2O_2 favors the activating phosphorylation of the IKK complex by inducing protein kinase D activity through Src-mediated signaling pathways[37] and mitogen-activated protein kinase kinase kinase (MAP3K).[38] Additionally, H_2O_2 can maintain high levels of activated IKK complexes through inactivation of protein phosphatases by oxidative modifications.[39] Finally, in agreement with the increased ratio p-IκBα/IκBα and the lower IκBα protein expression found in the F group, it has been postulated that H_2O_2 induces IκBα phosphorylation at several tyrosine residues, favoring its proteasomal degradation.[40,41]

Overall, the present results further describe the mechanisms associated with the prevention of the deleterious effects of high fructose feeding by (−)-epicatechin supplementation. In addition, this study supports the potential use of (−)-epicatechin-enriched foods or supplements as a nutritional tool to help down-regulate redox-sensitive pro-inflammatory conditions such as those found in diabetes, hypertension and dyslipidemia.[42,43]

Table 1 Changes in the expression of NOX subunits by high-fructose and (−)-epicatechin supplemented diets in rat kidney cortex

	Protein expression (fold increase with respect to untreated controls)	
	F	FE
NOX2[a]		
Catalytic subunit ($gp91^{phox}$)	1.5	1.5
Activator subunit ($p47^{phox}$)	1.3	1.0
NOX1		
Catalytic subunit (NOX1)	1.0	1.0
Activator subunit (NOXO1)	1.9	1.9
NOX4		
Catalytic subunit (NOX4)	1.6	1.0

F: fructose and FE: fructose-(−)-epicatechin groups. [a] Taken from ref. 8.

Abbreviations

IκBα	Inhibitor of κBα
NF-κB	Nuclear factor κB
NOX	NADPH oxidase
NOXO1	NOX1 organizer
TLR-4	Toll-like receptor 4

Conflicts of interest

There are no conflicts of interest to declare.

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