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The Protective Effect of Apocynin on Cyclosporine A-Induced Hypertension and Nephrotoxicity in Rats

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ABSTRACT

Cyclosporine A (CsA) is the prototype of immunosuppressant drugs that has provided new perspectives in human and veterinary medicine to prevent organ transplant rejection and to treat certain autoimmune diseases and dermatologic diseases. Unfortunately, the treatment with CSA is often limited by severe adverse effects such as hypertension and nephrotoxicity. Some data suggest that reactive oxygen species (ROS) and the oxidative stress play an important role in its pathogenesis, in particular the superoxide (O_2^-) that is the most powerful free radical generated by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase present mainly in the kidney. The present study has been designed to investigate the role of Apocynin a selective inhibitor of NADPH oxidase activity on cyclosporine-induced adverse effect. In this study, we have evaluated the effect of CsA, used alone or in association with Apocynin on blood pressure (BP), on glomerular filtration rate (GFR), on absoluted fluid reabsorption (Jv) in proximal tubule (PT), on O_2^- concentration, and on nitric oxide (NO) production. We have demonstrated that CsA administration increases superoxide concentration in the aorta, decreases the NO concentration, reduces GFR and the Jv in PT, and induces a significant increase in BP. Moreover, we have shown that Apocynin treatment restores these hemodynamic alterations, as well as NO and superoxide productions. In conclusion, the reported data indicate that CsA induced nephrotoxicity and hypertension are related to NADPH oxidase activity, in fact Apocynin protects the kidney function and BP from toxic effects induced by CsA through the inhibition of NADPH oxidase activity. J. Cell. Biochem. 116: 1848–1856, 2015. © 2015 Wiley Periodicals, Inc.

KEY WORDS: CYCLOSPORINE A; REACTIVE OXYGEN SPECIES; APOCYNIN; NITRIC OXIDE

The use of Cyclosporine A (CsA) has provided new perspectives in human medicine to produce immunosuppression in organ transplant recipients and in autoimmune diseases, in veterinary medicine to prevent certain dermatologic diseases on small animals [Robson and Burton, 2003; Colombo and Ammirati, 2011]. Unfortunately, its clinical use is limited by several side effects such as nephrotoxicity and hypertension. Two forms of nephrotoxicity induced by CsA have been described: acute and chronic. In acute nephrotoxicity induced by CsA, reversible reduction of glomerular filtration rate (GFR) and renal blood flow

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have been observed [Barros et al., 1987]. In chronic nephrotoxicity induced by CsA treatment, these hemodynamic alterations are irreversible and could be caused by vasoconstriction of the glomerular afferent arterioles [Nankivell et al., 2003].

The pathogenesis of hypertension is not completely clear. There is evidence suggesting that the retention of sodium and water is related to the development of hypertension induced by CsA [Ciresi et al., 1992], and the free radical involvement has been hypothesized [Krauskopf et al., 2002; Damiano et al., 2015]. Activation of the renin–angiotensin–aldosteron system (RAAS) seems to play a pivotal role in the hypertension induced by CsA. The mechanism responsible for angiotensin II (Ang II)-induced hypertension is very various [Lassila, 2002]. It is possible that the RAAS acts on GFR by constricting the efferent glomerular arterioles. However, recent studies have evidenced a role of reactive oxygen species (ROS) in the pathogenesis of Ang II-dependent hypertension during CsA treatment [Nishiyama et al., 2003]. ROS play a role as initial triggers for the direct or indirect modulation of different second messengers necessary for vasoconstriction. It is possible that overproduction of ROS during CsA treatment can lead to oxidative stress that causes decrease of the nitric oxide (NO) signaling pathway [Forstermann, 2010]. NO has anti-inflammatory and vasodilator functions, but under excessive concentration of ROS, limited presence of nitric oxide synthase (eNOS) could induce vasocostriction [Blough and Zafiriou, 1985; Gryglewski et al., 1986]. Among the ROS, superoxide concentration (O_2^-) is the most powerful which is mainly produced in the kidney by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [Wilcox, 2005]. NADPH-oxidase subunits are located in the kidney in interstitial cells, blood vessels, glomeruli, and tubules [Radeke et al., 1991] and play a crucial role in various signaling pathways leading to regulation of gene and protein functions under normal conditions of oxidative balance. It is hypothesized that to reduce the effects of NADPH oxidase during hypertension, a main focus should be the inhibition of its activity by blocking the assembly of the subunits of NADPH oxidase, the electron transfer, and consequently, the ROS production [Selemidis et al., 2008]. Apocynin, also known as acetovanillone or 4-hydroxy-3-methoxy acetophenone, is the best known inhibitor of NADPH oxidase. It was found that it acts blocking the interaction of p47phox with p22phox subunit of NADPH oxidase complex [Johnson et al., 2002].

In the present paper, we have investigated the involvement of O_2^- and NO in CsA hypertensive rat through the block of the NADPH oxidase activity by treatment with Apocynin. We have analyzed the alteration of the blood pressure (BP), of the GFR through the clearance of inulin, and we have evaluated the alteration of absoluted fluid reabsorption (Jv) in proximal tubule (PT) by in vivo micropuncture study. We have also analyzed the renal concentration of O_2^- by dihydroethidium (DHE) assay and NO concentration by nitrite and nitrate assay. The kidney tissue injury was analyzed by measuring of fibronectin abundance.

MATERIALS AND METHODS

The rats were obtained from Harlan Laboratories Srl (San Pietro al Natisone, Udine, Italy); Cyclosporin A was provided from Novartis Farma S.p.A. (Correggio, Varese, Italy); and Apocynin was obtained from Sigma (Milan, Italy).

ANIMAL PREPARATION

Experiments were performed on a total of 75 adult male Sprague–Dawley rats weighing 200–230 g. They were housed under constant environmental conditions (temperature 22°C and a 12-h light–dark cycle). Animals were fed a standard diet; food and water were given ad libitum. Rats were maintained in accordance with the guidelines of the NIH (Guide for the Care and Use of Laboratory Animals, 1996).

EXPERIMENTAL PROTOCOL

The rats were randomized and divided into five groups of 15 animals each and treated for 21 days as follows: the first group (control) received a daily equivalent volume of vehicle for CsA, castor oil, intraperitoneally (i.p.); the second group received a daily equivalent volume of vehicle for Apocynin, distilled water, orally (o.s.); the third group received CsA (15 mg/kg/i.p./die/2 ml); the fourth group received CsA (15 mg/kg/i.p./die/2 ml) and Apocynin (16 mg/kg/o.s./die/1 ml); and the fifth group received Apocynin (16 mg/kg/o.s./die/1 ml). BP was recorded in anesthetized rats, during the clearance and micropuncture experiments, through the carotid and femoral artery using a BP recorder (Pressure Monitor BP-1, Word Precision Instruments). The dose of CsA (15 mg/kg/day) administration is normally accepted to induce acute or chronic nephrotoxicity on the basis of the time of treatment [Damiano et al., 2013]. The dose of Apocynin has been chosen on the basis of a previous in vivo study [Panico et al., 2009].

CLEARANCE OF INULIN

GFR was measured at the end of the experimental treatment. The rats were anesthetized with an intra-peritoneal injection of Inactin (Sigma–Aldrich, St. Louis, MO), 120 mg/kg^{-1} body weight, tracheostomized, placed on a thermo-regulated table (37°C), and prepared for renal clearance evaluation. In brief, the right carotid artery was catheterized to monitor BP through a BP recorder (BP1 by WPI, USA) and to take blood samples for inulin concentration measurements. The left jugular vein was cannulated with polyethylene PE-50 tubing and used for intravenous infusion via a syringe pump (Braun, Melsungen) of 0.74 mg/100 g bw/min inulin from 10% saline solution. The surgical procedure also included bladder catheterization with PE-205 tubing. After a 60-min equilibration period, the first of four 30-min urine collections began. Arterial blood samples (100 μl) were taken at the beginning and end of each collection period. Inulin concentrations in plasma and urine were measured by the colorimetric method, and the absorbance was read at 550 nm using a Perkin Elmer UV Spectrophotometer. The GFR may be calculated as: (perfusion rate $\times$ perfusate inulin)/(plasma inulin) where perfusate inulin and plasma inulin are the inulin concentrations in perfusate and plasma, respectively.

PROXIMAL TUBULE MICROPUNCTURE

Each animal was anesthetized with inactin 120 mg/kg^{-1} body weight administered by i.p. injection, tracheotomized, and placed on a heated and thermostatically controlled (37°C) micropuncture table. The right femoral artery was then cannulated with

polyethylene tubing (PE 50) connected to the BP transducer for continuous monitoring. A second polyethylene tube was placed in the left jugular vein and connected to an infusion pump (2Biol Instruments) that delivered a modified Ringer's solution (NaCl 125 mmol/L^{-1}; $NaHCO_3$ 25 mmol L^{-1}) at a rate of 1.8 mL h^{-1}. The left kidney was then exposed through a flank incision, cleared of perirenal fat, and immobilized in a plastic kidney cup with agar (3% in saline), and then bathed with warmed mineral oil. Thirty to sixty minutes was allowed after surgery before beginning tubule micropuncture.

The PT site was identified by injections from a "finding" pipette containing dye-stained artificial tubular fluid (ATF). The flow was blocked by injection of T grease (T grade, Apiezon Products, Manchester, UK) via a micropipette (tip diameter 10–12 μm) proximal to the perfusion site.

The tubule was perfused with a micropipette (tip diameter 8–10 μm) connected to a microperfusion pump (model A1400, World Precision Instruments, Inc, Sarasota, FL) at 20 ± 3 nl/min. The perfusion solution contained FITC inulin as volume marker and 0.1% FD&C green dye for identification of the perfused loops. Tubule fluid collections were made at a downstream site with a micropipette (7–10 μm) following placement of a column of oil to block downstream flow. Samples were collected for 3–5 min and transferred into a constant-bore capillary tube whose length was measured with a micrometer to calculate the tubular fluid volume. Thereafter, collected samples with less than 90% and more than 110% of microperfused inulin were discarded. To determine the lengths of the perfused segments, tubules were filled with high-viscosity microfil (Flow Tech, Inc. Carver, MA). At the end of the experiments, the kidney was partially digested in 20% NaOH, and the casts were measured under a dissecting microscope. The Jv was calculated by the difference in the perfusion rate and the collection rate factored by the length of the nephron: $Jv = V_{perf}$ (nl/min)$-V_{coll}$ (nl/min)/PT length (mm) and expressed nl/min/mm. The composition of the perfusion fluid was as follows in mM: 125 NaCl, 20 $NaHCO_3$, 5 KCl, 1 $MgSO_4$, 2 CaCl, 1 $NaHPO_4$, 5 glucose, and 4 urea. At the end of each experiment, tubular fluid samples were transferred individually to 0.5 μl constant-bore Microcaps to determinate the volume. Then FITC-labeled inulin fluorescence was determined in samples and standards using a Spectra Max2 plate reader with excitation and emission wavelengths of 490 and 520 nm, respectively, according to Lorenz and Gruenstein [1999].

SUPEROXIDE ASSAY IN THE DISSECTED AORTA

Superoxide concentrations within the cells of the abdominal aorta were evaluated by the oxidation of dihydroethidium (DHE; Molecular Probes). DHE can enter the cell and be oxidized by superoxide to yield ethidium (Eth), which binds DNA producing bright red fluorescence. The increase in Eth-DNA fluorescence indicates peroxide production within cells [Carter et al., 1994]. DHE assay is specific to quantify the O_2^- production [Fink et.al., 2004].

The aorta was dissected and frozen at −80°C. Sections (10-μm thick) were cut at the cryostat (Leica CM 1850) and then incubated with a 20 μM DHE solution for 30 min at room temperature. Cover slips were mounted with Dako Fluorescence Mounting Medium.

Pictures were acquired by a Leica DMI 6000 B inverted microscope. Fluorescence intensity (IF) was quantified by Image-J software. Briefly, average pixel intensity was quantified from three equal random areas from each sample. The average pixel intensity was compared among groups. The data were expressed as IF/g of tissue × micrograms of proteins.

NITRITE AND NITRATE ASSAY

The production of nitrite (NO_2) and nitrate (NO_3), stable metabolites of NO production, was determined in the supernatant of kidney cytosols by Griess reagent. Nitrate was reduced to nitrite by addition of nitrate reductase (0.4 U/ml) in the presence of 10 mM NADPH and 2.5 mM flavin adenin dinucleotide and then assayed as nitrite. The plates were incubated with the Griess reagent at 25°C under reduced light for 20 min. Absorbance was read at 550 nm using a Perkin Elmer UV Spectrophotometer. The concentration of NO_2 was calculated on a calibration curve (range: 0.125–16 μg/ml), prepared using dilutions of sodium nitrate in the plating medium. The data were expressed as picomoles of nitrite for milligrams of proteins.

WESTERN BLOT ANALYSIS

Western blot or immunoblotting allows investigators to determine, with a specific primary antibody, the relative amounts of the protein present in different samples. Samples, prepared from rat renal cortex, were homogenized with Polytron in a lysis buffer composed of 0.3 M sucrose (SIGMA), 2 μM leupeptin (Sigma), 1 mM phenylmethane-sulphonylfluoride (PMSF, Sigma), 25 mM imidazole (Sigma), and 1 mM EDTA (Sigma) which protects proteins of interest from degradation. The protein concentration was determined by Bradford Assay, reading each sample at spectrophotometer (Biorad) at 595 nm, absorbance proportional to the amount (concentration) of protein present in the sample. The samples were loaded (30 μg) on NuPage Bis-Tris gel 4–12% (Novex by Thermo Fisher Scientific Inc.) with sample reducing buffer 10X (Invitrogen) and four X LDS NuPAGE Sample Buffer (Invitrogen) after heating at 70°C for 15 min. The 20X Mops SDS Buffer (Novex by Thermo Fisher Scientific Inc.) and NuPAGE Antioxidant (Invitrogen) were used for electrophoretic separation of proteins at 200 V in a Novex Mini-cell electrophoresis chamber (Invitrogen) for 60 min. Later, proteins were transferred to Invitrolon PVDF filter paper sandwich (0.4 μm pore size, Invitrogen), activated with methanol for 5 min using 20X Transfer Buffer NuPAGE (Novex by Thermo Fisher Scientific Inc.) for 70 min at 30 V. The PVDF membrane was incubated with casein I-block (Tropix) for 1 h and then with the Fibronectin primary antibody (Santa Cruz) and β-actin primary antibody (Santa Cruz) diluted 1:100 and 1:1,000 in I-block, respectively. The membrane was incubated overnight at 4°C. The following day, the blot was washed thrice with I-block for 5 min each after which it was incubated with secondary antibody–enzyme anti Mouse conjugated to alkaline phosphatase (Applied Biosystems) for both Fibronectin (1:1,000) and β-actin (1:4,000) for 1 h. The membrane was washed again as mentioned above and incubated with Western Light Plus Kit, CSPD and Nitroblock (Tropix), for 5 mins to develop the chemiluminescent signal. Protein expression levels were quantitatively estimated by densitometry using a Gel Doc scanner (BioRad) equipped with a densitometric workstation. The protein concentrations were normalized to the actin level and expressed as the densitometric ratio.

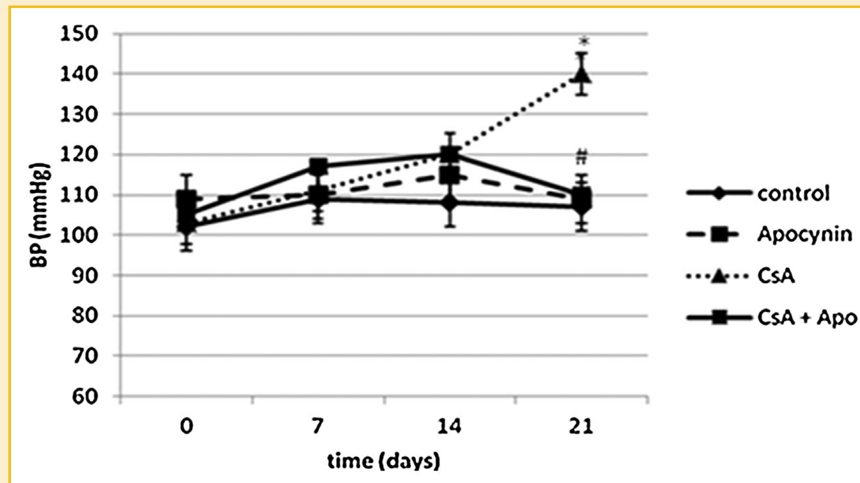

Fig. 1. Effects of CsA and Apocynin used alone or in association on blood pressure (BP) in rats treated for 3 weeks. CsA treatment significantly increased BP, while co-administration with Apocynin restored this effect (values mean ± SD; $^{*}P < 0.05$ vs. control group, $^{\#}P < 0.05$ vs. CsA group).

STATISTICAL ANALYSIS

All data are mean ± SD. Statistical analysis was performed by one-way ANOVA followed by the unpaired *t*-test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

BLOOD PRESSURE

As shown in the Figure 1, CsA increased BP, expressed as mmHg, at 21 days of the treatment compared to control. In fact, BP values shifted by 103 ± 12.01 control to 140 ± 5.37 CsA, respectively (n = 6; $^{*}P < 0.05$ respect to control). Apocynin, utilized alone did not exert any change (121 ± 2.98). However, the administration of Apocynin to animals treated with CsA restored this function (112 ± 14.56 Apocynin, $^{*}P < 0.05$ respect to CsA 140 ± 5.37). Only after 21 days of treatment, the effect on BP was very significant. Therefore, according to these results, we have focused our attention to the 21st day of treatment.

CLEARANCE OF INULIN

Figure 2 shows the CsA effect on renal hemodynamics. GFR was measured by means of inulin clearance and expressed as ml/min 100 g bw. CsA treatment significantly decreased GFR compared to

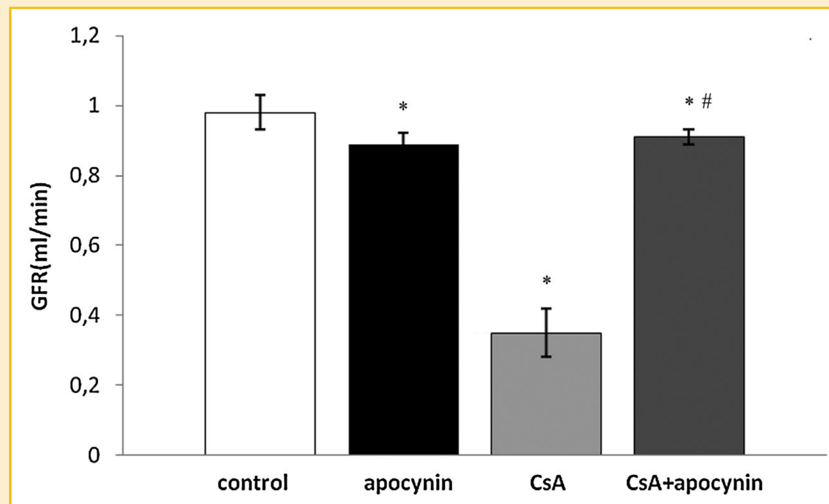

Fig. 2. Effects of CsA and Apocynin used alone or in association on glomerular filtration rate (GFR) after 3 weeks of the treatment. CsA treatment significantly decreased GFR while co-administration with Apocynin restored this effect (values mean ± SD; $^{*}P < 0.05$ vs. control group; $^{\#}P < 0.05$ vs. CsA group).

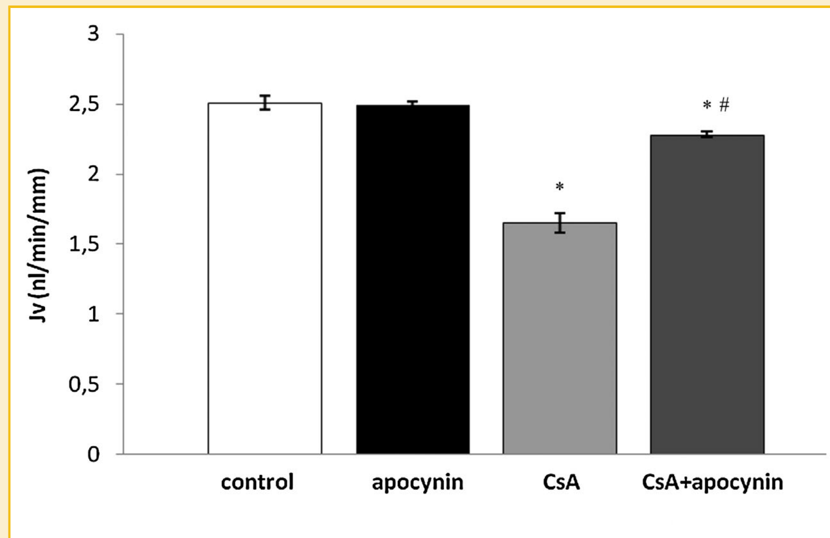

Fig. 3. Effects of CsA and Apocynin on absoluted fluid reabsorption (Jv) in rats treated after 3 weeks of the treatment. CsA treatment significantly decreased Jv while co-administration with Apocynin restored this effect (values mean ± SD; $^{*}P < 0.05$ vs. control group; $^{\#}P < 0.05$ vs. the CsA group).

control animals (0.35 ± 0.07 vs. 0.98 ± 0.05, respectively; $^{*}P < 0.05$ respect to control), and Apocynin, utilized alone, did not exert any change on glomerular function (n = 6; 0.89 ± 0.03 respect to control 0.98 ± 0.05). Also, the saline solution, used to dissolve Apocynin, when utilized alone, did not exert any change (data not shown); while treatment with Apocynin in association with CsA, exerted a good protection on CsA-induced GFR reduction (0.91 ± 0.02, $^{\#}P < 0.05$ respect to CsA 0.35 ± 0.07).

MICROPUNCTURE EXPERIMENTS

Jv, expressed as nl/min/mm, in the PT of control rats averaged 2.51 ± 0.23 (Fig. 3). However, Jv measured in the PT of rat treated with CsA was 1.65 ± 0.23 ($^{*}P < 0.05$ respect to control). Apocynin, utilized alone, did not change the Jv (2.49 ± 0.22). Treatment with Apocynin plus CsA restored the Jv levels (2.28 ± 0.32, $^{\#}P < 0.05$ respect to CsA).

SUPEROXIDE ASSAY

The O_2^- production was evaluated by DHE assay. As expected, CsA-treated rats presented a larger fluorescence intensity than controls (60,562 ± 7,370 and 42,074 ± 5,685, respectively; $^{*}P < 0.05$). Apocynin, utilized alone, did not change the O_2^- production (52,008 ± 1,958). Whereas co-administration of both CsA and Apocynin partially reversed the O_2^- production to the control level (51,552 ± 1,789; Fig. 4, panels A and B).

NITRITE AND NITRATE ASSAY

As shown in the Figure 5, CsA decreased the NO production (expressed as pmoles/mg of proteins) in rat kidney cytosol respect to control (10.23 ± 0.8 CsA respect to 23.11 ± 1.0 control, $^{*}P < 0.05$). Apocynin, used alone, did not show any significant difference respect to control (22.29 ± 0.9 and 23.11 ± 1.0, respectively). Treatment with Apocynin plus CsA restored the levels of NO concentrations respect to CsA group (19.68 ± 0.8, $^{\#}P < 0.05$ respect to CsA).

WESTERN BLOTTING

The renal injury was evaluated using western blotting to determine fibronectin abundance, and the results are shown in Figure 6. A protein with a molecular mass between 220 kDa and 250 kDa was recognized by the antibody. Densitometric analysis demonstrated that fibronectin abundance, normalized for β-actin, increased in CsA animals compared to control animals (1.6 ± 0.10 CsA respect to 1.1 ± 0.09 control). Apocynin, utilized alone, did not change this value (1.2 ± 0.07).

This value decreased when animals were treated with Apocynin plus CsA (0.81 ± 0.11).

DISCUSSION

Several studies indicate that the clinical use of CsA is limited since the hypertension and kidney dysfunction was observed during long- and short-term treatment with this drug. Previous studies showed that long treatment with CsA could be the cause of chronic CsA nephrotoxicity (CCN) [Montagnino et al., 2004; Magnasco et al., 2008; Damiano et al., 2013], and other reports have shown that the CsA chronic treatment in the rats induced similar effect to those reported in humans [Ryffel et al., 1986; Settaf et al., 1989].

For this reason, the rats animal models have been chosen to study the CCN in humans and veterinary medicine. In the present study, in agreement with our previous investigation [Damiano et al., 2013], we have demonstrated that chronic treatment with CsA significantly increase the BP after 21 days of treatment at the dose of 15 mg/kg/i.p./die/2 ml (Fig. 1). Several researchers suggest an important role of oxidative stress in CsA induced hypertension and renal failure [Tufro-McReddie et al., 1993; Damiano et al., 2013], but the mechanism by which CsA causes these effects is still unclear.

In the present study, we have shown that chronic treatment with CsA for 21 days significantly increase the BP and induce severe

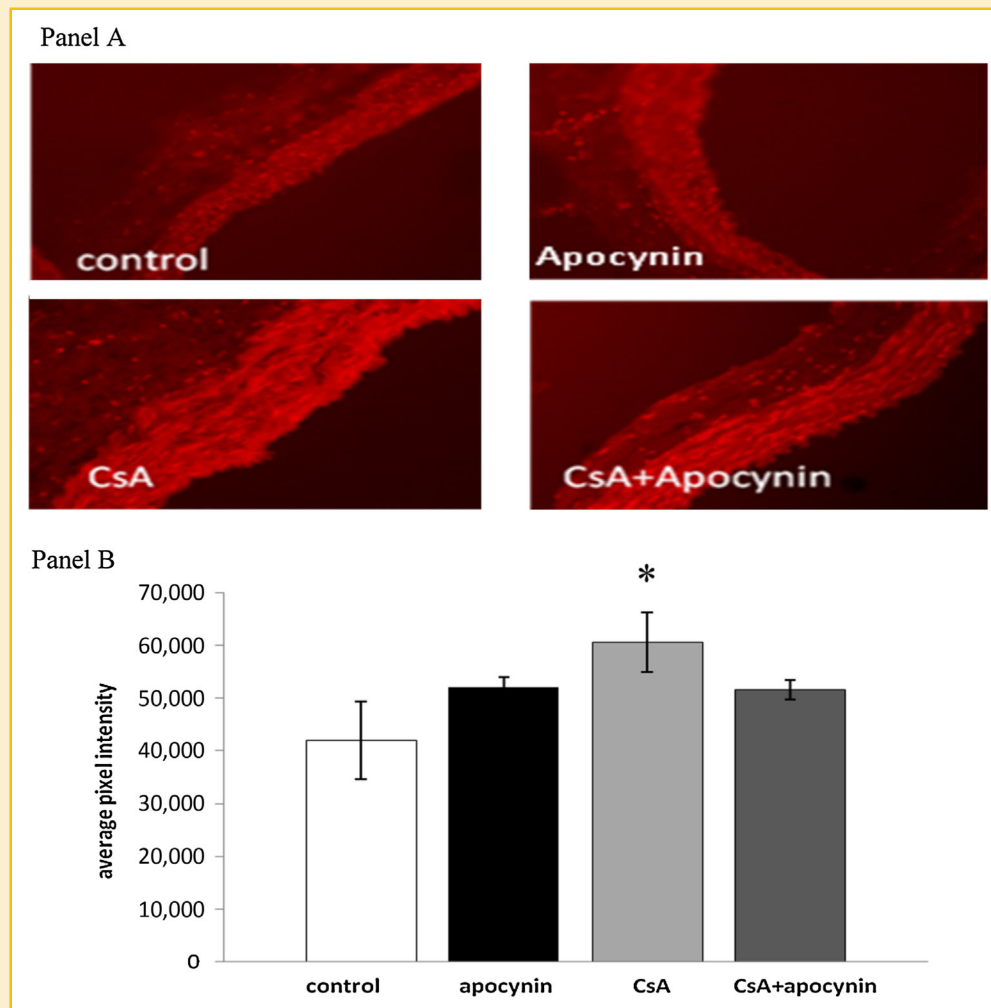

Fig. 4. Effects of CsA and Apocynin on O_2^- production by dihydroethidium (DHE) assay. Panel A: Representative pictures of DHE staining on the rat aorta in rats after 3 weeks of the treatment. CsA treatment induces an increase of DHE fluorescence signal as the expression of superoxide-mediated intracellular injury. Apocynin prevents CsA-induced injury on the aorta. Panel B: Fluorescence intensity quantification. Magnification × 200 (values are mean ± SD), fluorescence intensity was quantified by Image-J software. Briefly, average pixel intensity was quantified from three equal random areas from each sample (*$P < 0.05$ vs. control group).

alterations of renal hemodynamics, such as a decrease of GFR (Fig. 2) and in JV in PT (Fig. 3). In addition, we also demonstrated by measuring the fibronectin abundance, a severe kidney injury (Fig. 6).

A pivotal role in this pathophysiologic process seems to be done by the fibrosis and vasoconstriction, and in particular, an imbalance between vasoconstrictors and vasodilators has been hypothesized [Wang et al., 2003]. Among vasodilating compounds, a decrease in circulating NO has been proved to be particularly relevant in producing vasoconstriction and hypertension. It has been proposed that the inhibition of NO synthesis is related to the overproduction of free radicals induced by CsA with the consequent manifestation of hypertension. It is also suggested that the inhibition of NO synthesis is related to the increase of vascular superoxide formation mediated by Angiotensin II (Ang II) that stimulate NADPH oxidase to produce more O_2^- that, rapidly, is converted to other ROS [Lopez-Ongil et al., 1998]. In this paper, we have investigated, in CsA hypertensive rats, the link between the production of O_2^- and NO, by measuring the formation of O_2^- in the aorta artery by DHE experiments and the production of NO by nitrite and nitrate assay, and we have found that the increased production of O_2^- (Fig. 4) is related to the reduction in the concentration of NO (Fig. 5). This link between O_2^-/NO is most probably the first step that triggers the process of hypertension and functional alteration. In fact, it has been demonstrated that NO promotes fluid reabsorption in PT [Wang, 1997] and that the reduction of Jv in PT could be compensated by the reduction in GFR through TGF activation. It has been suggested that the cells of the macula densa of the juxtaglomerular apparatus sense the osmolarity of the tubular fluid and convert it into a signal which affects the GFR [Wang, 1997].

During the treatment with a specific inhibitor of NADPH oxidase, Apocynin, we have observed a recovery of O_2^- and NO productions to the balance value, and simultaneously, we have observed a restore of BP and renal hemodyamic. In this way, we have shown a strong link between high BP and fall of GFR and that these changes are linked to an increase of NADPH oxidase activity that stimulates the

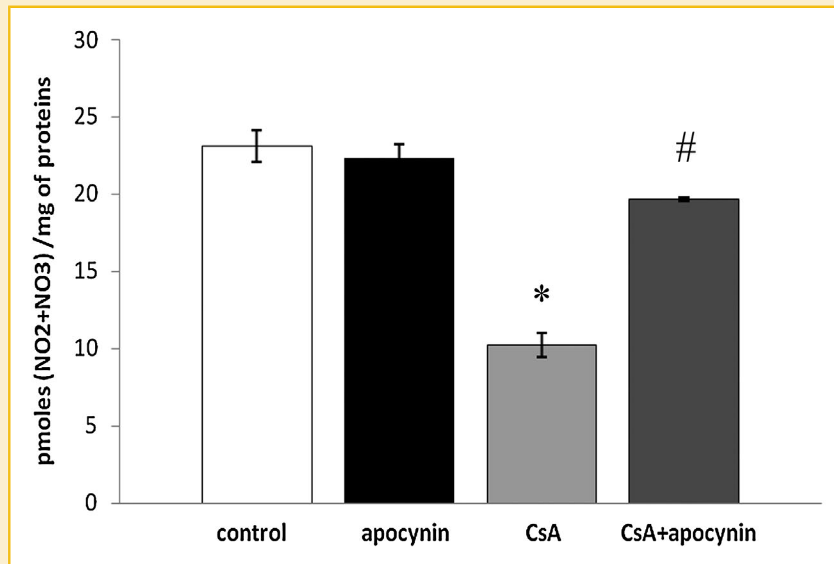

Fig. 5. Effect of CsA and Apocynin on nitric oxide (NO) production from renal cell of rat after 3 weeks of the treatment measured by Griess reagent. NO production is reduced in CsA-treated rats. Apocynin restored this effect ($^{*}P < 0.05$ vs. control group, $^{\#}P < 0.05$ vs. the CsA group).

overproduction of O_2^- which could be the cause of NO reduction bioavailability. We have also demonstrated, by measuring of fibronectin abundance (Fig. 6), that the kidney injury observed during CsA treatment, was completely restored in rats treated with Apocynin plus CsA. Fibronectin in extracellular matrix during tubule interstitial fibrosis acts as a protein scaffold for the deposition of other extracellular matrix proteins [Gharaee-Kermani et al., 1996]. Furthermore, the fibronectin scaffold has been demonstrated to be involved in the differentiation of fibroblasts during renal fibrosis [Serini et al., 1998]. Our study have evidenced, through

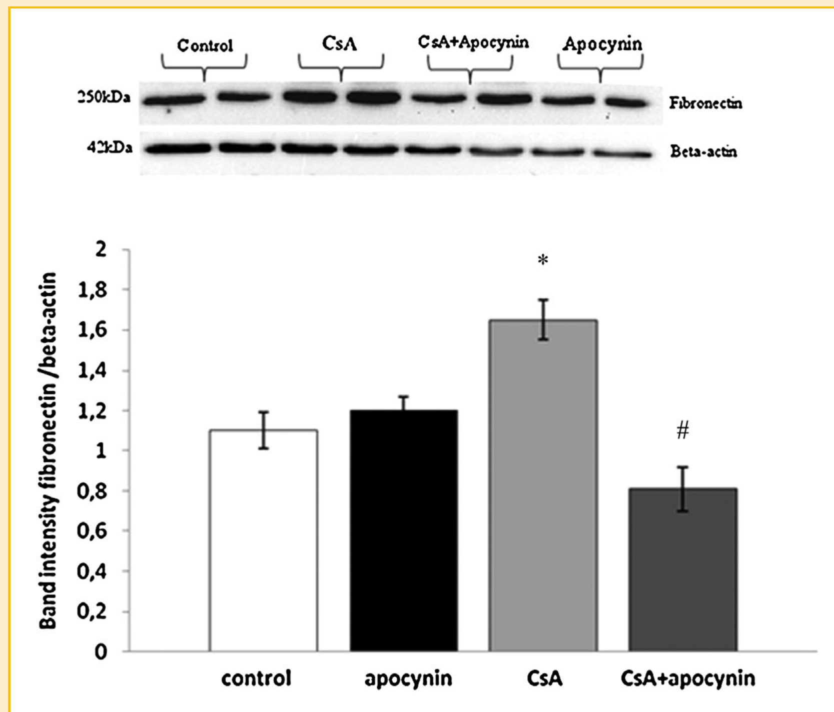

Fig. 6. Effect of CsA and Apocynin on fibronectin abundance of rat after 3 weeks of the treatment. Normalization of fibronectin with the β-actin and quantification by the image J program ($^{*}P < 0.05$ vs. control group, $^{\#}P < 0.05$ vs. the CsA group).

fibronectin abundance, during CsA chronic renal injury is in agreement with the previous hypothesis that a pivotal role in the pathophysiologic process induced by CsA is made by the fibrosis and vasoconstriction [Tufro-McReddie et al., 1993].

In conclusion, our data suggest that at the root of the hypertensive process during the treatment with CsA, there is an increase in NADPH oxidase activity, and consequently, the O_2^- productions. When we have blocked the activity of NADPH oxidase by treatment with Apocynin, we have shown the restore of the kidney function with a decrease of BP. Among the antioxidants previously used by us [Galletti et al., 2005; Capasso et al., 2008; Damiano et al., 2013], Apocynin is the first one that simultaneously restores the BP and kidney function. Probably, this effect is related to different mechanism of the action of the antioxidant used. In fact, the previously used drugs, such a hydrocortisone, hydroxytyrosol, and the recombinant mitochondrial manganese-containing superoxide dismutase (rMnSOD), acted on all types of ROS. On the contrary, using Apocynin, we have shown that only blocking the O_2^- produced by NADPH oxidase, it is possible to completely restore kidney function and BP simultaneously.

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