The Distinct Alterations Produced in Cardiovascular Functions by Prednisolone and Nitro-prednisolone (NCX-1015) in the Rat Highlight a Causal Role for Endothelin-1

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ABSTRACT

Daily administration of prednisolone, but not the derivative NCX-1015 (or prednisolone 21-[4’-nitrooxymethyl]benzoate), to rats resulted in a time- and dose-dependent increase in mean arterial blood pressure (MABP), significant after 1 week for the lower dose of 1.38 μmol/kg i.p. and after 3 weeks for the higher dose of 6.9 μmol/kg i.p. A similar dichotomy of behavior was observed with respect to myocardial contractility and renal vascular resistance, in either case augmented by 3-week treatment with prednisolone but not NCX-1015. In contrast, both NCX-1015 and prednisolone reduced plasma levels of corticosterone in a dose- (dose range of 0.69–6.9 μmol/kg) and time-dependent (1–3 weeks) manner. Similar profiles were obtained for plasma nitrate values, although they were increased selectively after NCX-1015 administration. In contrast, prednisolone, but not NCX-1015, augmented plasma endothelin 1 (ET-1) with a profile that mirrored the changes observed in MABP and renal blood flow. Supply in the drinking water of the ET-1 receptor type A (ET_{A}) antagonist FR139317 ([R-2-[(R)-2-[(S)-2-[(1-hexahydro-1H-azepinyl)-carbonyl]amino-4-methylpentanoyl]-amino-3-(2-pyridil)propionic] or mixed ET_{A/B}, but not of selective ET_{B} antagonists prevented the changes produced by a 21-day treatment with prednisolone. In conclusion, this study indicates 1) a lack of occurrence of cardiovascular alterations by nitro-releasing derivative of prednisolone (NCX-1015), and 2) a functional link between prednisolone effects and the endogenous endothelin-1 system.

Glucocorticoids (GCs) have wide clinical applications for the management of a variety of disorders, including autoimmune, allergic, and lymphoproliferative diseases. In most of these pathologies, GC must be administered for long term, and this increases the likelihood of the appearance of major side effects, thus limiting further use (Whitworth, 1994; Saruta, 1996). Marked side effects include obesity with the classical “moon face”; hirsutism; cataracts; osteoporosis; diabetes mellitus; immune suppression; and cardiovascular disorders, including hypertension and atherosclerosis (Ross and Linch, 1982; Schäcke et al., 2002). Among these multiple effects, cardiovascular complications are an important factor for predicting the morbidity and mortality of patients over-treated with GCs (Ross and Linch, 1982). Plasma volume expansion due to sodium retention gives a minor contribution to GC cardiovascular effects (Whitworth et al., 1989; Whitworth, 1994; Saruta, 1996). In contrast, increase in peripheral vascular resistance, demonstrated by an augmented pressor response to catecholamines and angiotensin II, is a major contributor to the pathogenesis of hypertension after excess use of GCs (Pirpiris et al., 1992; Whitworth, 1994; Saruta, 1996). The molecular mechanism whereby GC excess causes the increase in vascular resistance and hypertension has not been fully elucidated, although a recent study has provided a functional link between dexamethasone administration and down-regulation of endothelial nitric-oxide (NO) synthase (Wallerath et al., 2004).

Vascular tone is regulated by the release of several relaxing and contracting factors that modulate the contractile...
activity of vascular smooth muscle cells (Lüscher and Barton, 1997; Weber et al., 1998). Among these factors a major function is played by prostanooids and NO. Endothelial cell-derived NO (Furchgott and Vanhoutte, 1989) is able to produce vascular relaxation, and a reduced NO availability (i.e., due to perturbation of its synthesis and/or release by vascular endothelial cells) causes a prompt increase in vascular resistance (Panza et al., 1993; Luscher and Barton, 1997).

Vascular resistance, blood pressure, and cardiac function are also increased after activation of the endothelin system (Ishikawa et al., 1988; Yanagisawa et al., 1988). Endothelin 1 (ET-1) is a 21-amino acid-long potent vasoactive peptide (Provencher et al., 1998). This peptide acts in strict cooperation, and in an antagonistic manner, with the NO system to produce a fine regulation of the degree of vessel dilation/contraction (Schini and Vanhoutte, 1991). Thus, an imbalance between these two vasoactive factors is believed to have a role in hypertension, left ventricular dysfunction, and cardiac hypertrophy pathologies (Schini and Vanhoutte, 1991; Kojda et al., 1997; Rothermund et al., 2000). Some of these pathological conditions are indeed characterized by an augmented ET-1 synthesis or release (Schiffrin et al., 1997; Ruscitzka et al., 2001). In addition, in different models of experimental hypertension (including deoxycorticosterone acetate-salt rats, Dahl salt-sensitive rats, and stroke-prone spontaneously hypertensive rats), ET-1 overexpressed in the vasculature has been reported (for review, see Iglarz and Schiffrin, 2003). The same applies in the clinical settings, because patients affected by moderate-to-severe hypertension present increased vascular endothelial and smooth muscle cell levels of prepro-ET-1 mRNA (Iglarz and Schiffrin, 2003).

Because GCs are the most potent anti-inflammatory agents available to date, their therapeutic use would generally benefit from a reduced burden of side effects, particularly those affecting the bone and the cardiovascular compartment. Recently a new GC, nitro-prednisolone, referred to as prednisolone 21-[[4-[(1,3-dihydro-2H-1,3-benzoxazin-2-yl)-methyl]phenyl]-2,6-dimethyloximate]benzoate or NCX-1015, has been described to release NO and nitrate species in biological fluids (Paul-Clark et al., 2000). NCX-1015 was found to be more potent than the parent compound prednisolone in in vitro assays of GC-receptor activation (Paul-Clark et al., 2003), and in a model of peritonitis it displayed more potent inhibitory effects on neutrophil extravasation, cytokine and chemokine release, and expression of proinflammatory enzymes (Paul-Clark et al., 2000). A subsequent study demonstrated a lack of osteoclast stimulating effect by NCX-1015, and this seemed to be genuinely due to NO released by the compound with activation of soluble guanylate cyclase in the target cell (Paul-Clark et al., 2002; Perretti et al., 2003).

The present study was undertaken to satisfy two major aims: to gain more information on mediator release in relation to long-term treatment with a GC (our choice was prednisolone for its diffuse clinical use) and insurgence of GC-induced major cardiovascular side effects; and to test whether the new GC NCX-1015, known to release nitrate species in biological fluids, would produce a profile of cardiovascular side effects similar to that displayed by prednisolone.

### Materials and Methods

#### Materials
NCX-1015 was synthesized at NicOx Research Institute (Milan, Italy) as described previously (Paul-Clark et al., 2000). Prednisolone was supplied by NicOx Research Institute, whereas FR139317 ([2R-(2R)-2-((3S)-2-[[1-(hexahydropyridin-2-yl)-azoni- myl]-4-methylpentanoyl]-amino-3-[4-pyridyl]propionyl]) was from Parke Davis Pharmaceutical Research (Ann Arbor, MI), SB209670 ([+x15,S2RS]+-2-carboxymethoxy-4-methoxyphenyl-1-3,4-methyl endoxygenoxyphenyl)-5-(prop-1-yloxy)indane-2-carboxylic acid) was from GlaxoSmithKline (Uxbridge, Middlesex, UK), and BQ-788 (N-cis-2,6-dimethylpiperedinocarboxyl-1-γ-metLeu-d-i-methoxy carbonylTrp-d-Nle) was from Banyu Pharmaceutical Co. (Tokyo, Japan).

#### Animals and Drug Treatment.
Male Sprague-Dawley rats (body weight ~150 g at the beginning of experimentation) were purchased from Charles River Italia (Milan, Italy). Rats were kept on standard chow pellet and had ad libitum access to water. Experiments were conducted upon authorization of Italian regulations on protection of animals used for experimental and other scientific purpose (D.M. 116192) as well as with the European regulations (O.J. of E.C. L 358/1 12/18/1986).

In total, 118 rats were used in the study. After recording of basal values (see below), animals were treated daily with a single dose of GC given i.p. Injections were done between 8:00 and 11:00 AM. The doses used were the following: prednisolone and NCX-1015 were given at 0.69, 1.38, and 6.9 μmol/kg to groups of 10 rats each. For prednisolone, these doses corresponded to 0.25, 0.5, and 2.5 mg/kg, respectively. A vehicle group in which animals were treated with peanut oil (0.5 ml/rat/day i.p.) was also added.

In the second set of experiments, rats were treated i.p. with 6.9 μmol/kg prednisolone with or without ET-1 receptor antagonists. The actual dose given for each antagonist was calculated according to the water intake measured three times weekly as described previously (Moreau et al., 1997). The average intakes of the ET-1 antagonists were 30 mg/kg/day for the selective type A (ETA) receptor antagonist FR139317 (Sogabe et al., 1993); 1 mg/kg/day for the selective type B (ETB) antagonist BQ-788; and 10 mg/kg/day for the nonselective ETα/ETβ antagonist (Ohlstein et al., 1994) SB209670. These doses are in line with those used in other studies addressing the effect of chronic treatment (Fujihara et al., 1995; Kozhukhi et al., 1998; Takeda et al., 1999; Iwasa et al., 2001). Antagonists were given over the entire period of treatment with prednisolone or with vehicle.

Body weight was recorded daily, whereas blood aliquots (0.5 ml) were taken by the tail vein after mild halothane anesthesia at the end of each week of treatment.

#### Determination of Systolic Blood Pressure.
Mean arterial blood pressure (MABP) was determined every week using a noninvasive methodology (tail cuff) as described previously (Bhanot et al., 1994; Galipeau et al., 2001). Briefly, rats were warmed in a heater for single holder LE 5610 (Leticia, Barcelona, Spain), housed singly in cages in an isolated room, and a cuff was placed around the tail artery. After a 15-min period of resting, MABP was recorded through an LE5200 apparatus (Leticia). Three measurements were taken at 5-min intervals, and the average of at least three measurements was obtained on each occasion (Galipeau et al., 2001).

#### Determination of Cardiac Function.
At the end of the treatment period (week 3 or 21 days), rats were anesthetized with inactin (100 mg/kg i.p.; RBI, St. Albans, UK), and the right carotid artery was catheterized for the measurement of MABP and +dP/dt. The latter parameter was evaluated by measuring the +dP/dt through a micropip pressure transducer catheter (model SPC-320; Millar Instruments Inc., Houston, TX; Sakai et al., 1996) inserted into the right carotid artery. +dP/dt was derived by active analog differentiation of the pressure signal by means of a Mac Lab system (ADInstruments, Hastings, UK). Heart rate was monitored by electrocar-
Effects of drug treatment on body weight

Rats were treated for 3 weeks with vehicle (peanut oil; 0.5 ml/rat/day i.p.), prednisolone, or NCX-1015 (0.69, 1.38, or 6.90 μmol/kg/day i.p. for either steroid). Data are values of body weight (grams) and are reported as mean ± S.E.M. of 10 rats per group.

<table>
<thead>
<tr>
<th>Experimental Group</th>
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<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
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<td>191 ± 3</td>
<td>207 ± 3</td>
<td>223 ± 3</td>
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<td>Pred 0.69</td>
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<td>207 ± 2</td>
<td>221 ± 4</td>
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<tr>
<td>Pred 6.90</td>
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<td>194 ± 3</td>
<td>208 ± 3</td>
<td>224 ± 3</td>
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<td>197 ± 4</td>
<td>203 ± 3</td>
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<tr>
<td>NCX-1015, 1.38</td>
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<td>196 ± 2</td>
<td>202 ± 3</td>
<td>225 ± 3</td>
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<tr>
<td>NCX-1015, 6.90</td>
<td>192 ± 3</td>
<td>197 ± 2</td>
<td>206 ± 3</td>
<td>224 ± 2</td>
</tr>
</tbody>
</table>

Determination of Renal Vascular Resistances. Once the animals were instrumented as described above, the left kidney was exposed via a mid-line laparotomy and the renal artery was isolated. An ultrasonic flow probe (internal diameter 1 mm), embedded in a silicone cuff to provide optimal alignment, was placed around the left renal artery to measure total renal blood flow (RBF) using a Transonic T206 flowmeter (Transonic Systems Inc., Ithaca, NY) as described by D’Amico et al. (1996). Renal vascular resistance (RVR) was then calculated as the ratio MABP/RBF.

Biochemical Analyses. After the completion of the hemodynamic measurements, a blood sample was taken. Aliquots of anticoagulated blood (5% EDTA) were centrifuged at 300g for 15 min. Supernatant (plasma) samples were stored at −20°C before analysis. Plasma ET-1 levels were quantified using a specific enzyme immunoassay (Cayman Chemical, Ann Arbor, MI), which shows high sensitivity (detection limit 1.5 pg/ml; data furnished by the manufacturer). Corticosterone was measured with an enzyme-linked immunosorbent assay kit (IDS, Boldon, UK) with a detection limit of 3.0 ng/ml. Levels of nitrate/nitrite were quantified with a commercial kit (R&D Systems, Abingdon, UK) which shows high sensitivity (detection limits were 0.22 μM for nitrite and 0.54 μM for nitrate; data furnished by the manufacturer).

Statistics. Data are reported as mean ± S.E.M. of 10 rats per group. Differences among experimental groups were analyzed by analysis of variance. An F value corresponding to a probability < 0.05 was required to reject the null hypothesis; intergroup variations were determined by Dunnett’s test (comparison versus the vehicle-treated group) or Bonferroni’s test (comparison between appropriate prednisolone and NCX-1015 groups). In either case, a P value < 0.05 was taken as significant.

Results

Effect on Body Weight. No significant changes in body weights were recorded across the treated experimental groups, compared either among themselves or with vehicle group (Table 1).

Effect on MABP. Basal MABP values around 110 mm Hg were measured in all animals before beginning of treatment. These values did not significantly change after i.p. administration of vehicle over the 3-week period (Fig. 1A). In contrast, treatment of rats with prednisolone caused significant alterations in MABP both in a dose- and time-dependent manner. For instance, daily treatment with the top dose used of 6.9 μmol/kg (corresponding to 2.5 mg/kg) significantly increase MABP after week 1, and values augmented thereafter to a maximum of 150 ± 17.7 mm Hg at week 3 (Fig. 1A). At the intermediate prednisolone dose of 1.38 μmol/kg (corresponding to 0.5 mg/kg), 3-week, but not 1- or 2-week, treatment was required to significantly alter MABP. The GC was inactive at the lowest dose tested of 0.69 μmol/kg (0.25 mg/kg) (Fig. 1A).

Importantly, equimolar doses of NCX-1015 failed to cause any significant changes in MABP from the vehicle-treated group (Fig. 1B). Comparison between the top doses of NCX-1015 and prednisolone showed significant difference at any week of treatment (P < 0.05 for week 1 and P < 0.01 for weeks 2 and 3; n = 10 rats).

Fig. 1. Effect of drug treatment on mean arterial blood pressure. A, rats were treated daily with vehicle (peanut oil; 0.5 ml/rat i.p.) or prednisolone (Pred; 0.69, 1.38, or 6.90 μmol/kg i.p.), and MABP was measured by tail cuff as described under Materials and Methods at weekly intervals. B, As in A, but NCX-1015 (NCX; 0.69, 1.38, or 6.90 μmol/kg i.p.) was given instead of Pred. Data are mean ± S.E.M. of 10 rats per group. *, P < 0.05 and **, P < 0.01 versus respective vehicle value.
Effect on Cardiac +dP/dt and Heart Rate. These parameters were measured at the end of the 3-week treatment with either prednisolone or NCX-1015. Figure 2 shows that 1.38 and 6.9 μmol/kg prednisolone increased cardiac contractility as measured by the augmentation in +dP/dt. This effect was significant for either dose, with the highest tested dose of 6.9 μmol/kg (2.5 mg/kg) giving a 72% ± 11% increment ($P < 0.01$ versus vehicle). In contrast, treatment of rats with equimolar doses of NCX-1015 failed to alter this parameter of cardiac function. For the top dose of 6.9 μmol/kg, a significantly lower +dP/dt value was measured ($P < 0.01$ versus equimolar dose of prednisolone; not significant versus vehicle). Neither GC nor dose treatment affected the heart rate values, compared with vehicle-treated control animals (Table 2).

Effect on Renal Vascular Resistance and Renal Blood Flow. Three-week treatment with prednisolone produced an increase in RVR and a corresponding decrease in RBF compared with vehicle-treated rats (Fig. 3, A and B). In contrast, no changes in either RVR or RBF were observed after chronic treatment with NCX-1015. At doses of 1.38 and 6.9 μmol/kg, RVR and RBF values for NCX-1015-treated rats were significantly different from the appropriate prednisolone group.

Biochemical Measurements in Rat Plasma Samples. Basal ET-1 immunoreactivity was detected in the plasma of vehicle-treated rats (Fig. 4, A and B). Prednisolone (1.38 μmol/kg or 0.5 mg/kg), administered for 3 weeks, significantly ($P < 0.05$) increased plasma ET-1 levels (Fig. 4A). If rats were treated with the top dose of 6.9 μmol/kg i.p. (equivalent to 2.5 mg/kg), plasma ET-1 increase was already evident after 1 week (Fig. 4B). At this dose, prednisolone produced almost a 3-fold increase in ET-1 levels ($P < 0.01$) compared with vehicle-treated rats, as measured at the 3-week time point (Fig. 4B). The lower dose tested of 0.69 μmol/kg i.p. (0.25 mg/kg) prednisolone did not modify plasma ET-1 content at any time (data not shown). Noteworthy, administration of equivalent doses of NCX-1015 to rats for 3 weeks did not produce any significant alterations in plasma ET-1 levels (Fig. 4, A and B).

TABLE 2

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Heart Rate (beats/min)</th>
</tr>
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<tbody>
<tr>
<td>Vehicle</td>
<td>368 ± 9</td>
</tr>
<tr>
<td>Pred, 0.69</td>
<td>361 ± 7</td>
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<tr>
<td>Pred, 1.38</td>
<td>382 ± 6</td>
</tr>
<tr>
<td>Pred, 6.90</td>
<td>362 ± 8</td>
</tr>
<tr>
<td>NCX-1015, 0.69</td>
<td>373 ± 6</td>
</tr>
<tr>
<td>NCX-1015, 1.38</td>
<td>373 ± 7</td>
</tr>
<tr>
<td>NCX-1015, 6.90</td>
<td>378 ± 7</td>
</tr>
</tbody>
</table>

Fig. 2. Effect of drug treatment on +dP/dt (derivative of pressure over time) values. Rats were treated daily with vehicle (peanut oil; 0.5 ml/rat i.p.), prednisolone (Pred; 0.69, 1.38, or 6.90 μmol/kg i.p.), or NCX-1015 (NCX, 0.69, 1.38, or 6.90 μmol/kg i.p.). After 3 weeks, +dP/dt (millimeters of Hg per second) was measured as described under Materials and Methods. Data are mean ± S.E.M. of 10 animals per group. *, $P < 0.05$ and **, $P < 0.01$ versus vehicle group; and #, $P < 0.05$ versus respective Pred group.

Fig. 3. Effect of drug treatment on renal blood flow and renal vascular resistances. A, rats were treated daily with vehicle (peanut oil; 0.5 ml/rat i.p.), prednisolone (Pred; 0.69, 1.38, or 6.90 μmol/kg i.p.), or NCX-1015 (NCX; 0.69, 1.38, or 6.90 μmol/kg i.p.). After 3 weeks, RBF was measured as described under Materials and Methods. B, as in A, but RVR is reported. In A and B, data are mean ± S.E.M. of 10 rats per group. *, $P < 0.05$ and **, $P < 0.01$ versus vehicle group; and #, $P < 0.05$ versus respective Pred group.

Basal levels for nitrate/nitrite and corticosterone were detected in the plasma of vehicle-treated animals, as reported in Table 3 and 4, respectively. Prednisolone (1.38 μmol/kg), administered for 3 weeks, mildly decreased circulating levels of both mediators. At the higher dose of 6.9 μmol/kg, prednisolone caused a significant decrease in circulating nitrate/
nitrite and corticosterone, with calculated of inhibition of 40 ± 2 and 73 ± 1%, respectively (Tables 3 and 4). Interestingly, whereas treatment of rats with NCX-1015 attenuated circulating corticosterone levels to a similar degree as prednisolone (Table 4), a marked increase in plasma nitrate/nitrite species was measured (Table 3). As for other measure-
ments, these parameters changed in a dose- and time-dependent manner.

**Functional Relationship between ET-1 and Cardiovascular Changes.** The second part of the study addressed the functional relationship between prednisolone and ET-1, because a positive correlation was found between the GC-induced changes in MABP and plasma ET-1 values (square coefficient of 0.976; Fig. 5A). Similarly, a significant correlation was found between the plasma levels of ET-1 and prednisolone-induced increase in RVR (Fig. 5B).

**Endothelin Receptor Antagonists: Effect on Prednisolone-Induced Cardiovascular Alterations.** Administration in the drinking water of FR139137 (30 mg/kg/day) to the rats greatly attenuated the increase in MABP induced by daily administration of prednisolone (6.9 μmol/kg) (Fig. 6A). Similarly, the increase in MABP induced by the GC was counteracted by administration of the mixed ET_A/ET_B receptor antagonist SB209670 (10 mg/kg) (Fig. 6A). In contrast, treatment of rats with the BQ-788, a selective antagonist at the ET_B receptor, produced a slight yet significant increase ($P < 0.05$) of MABP values produced by prednisolone both at week 2 and 3 (Fig. 6A). Given alone, the antagonists produced modest changes in MABP. Notably, FR139137 reduced MABP by ~10 mm Hg, whereas BQ-788 increased it by a similar degree (Fig. 6B).

The ET_A antagonist FR139137 was effective also in reducing prednisolone-induced increase in +dP/dt (Fig. 7A). The mixed antagonist SB209670 was less effective, whereas the selective ET_B antagonist BQ-788 worsened this parameter. The modest changes in MABP produced by these drugs alone (Fig. 6) were reflected in minimal alterations in +dP/dt (Fig. 7A). Heart rate values were unaffected by the GC, by ET antagonists, or their association when compared (Table 5).

This profile of effects was essentially retained when parameters of the renal vasculature were analyzed. In analogy to what was reported above (Fig. 3), 3-week treatment with prednisolone produced significant increases in RBF and RVR (Fig. 7, B and C): the selective ETA antagonist was active in preventing them, with SB209670 being equally effective, whereas BQ718 produced a modest augmentation of GC action (Fig. 7, B and C).

**Discussion**

In this study, we reproduced some of the cardiovascular alterations known to characterize prolonged systemic GC treatment and make two novel experimental contributions: first, an NO-donating GC derivative did not share the hypertensive property of prednisolone; and, second, a striking functional relationship between changes in MAPB and heart and renal functions with plasma ET-1 levels was found.

Clinical data indicate that hypertension, dyslipidemia, and a reduced fibrinolytic potential have been identified as the main cardiovascular adverse effects produced by GC (Sartori et al., 1999; Sholter and Armstrong, 2000). A recent meta-analysis of 163 severely asthmatic children receiving chronic oral or inhaled GC indicated an 88% incidence of hypertension (Covar et al., 2000). Equally important, in long-term stable renal and liver transplant patients treated with prednisolone, the GC dose was found to be the only independent variable to predict increased serum cholesterol levels (Fernandez-Miranda et al., 1998). In addition, a hypofibrinolytic
state due to increased plasminogen activator inhibitor-1 activity was reported in 69% of the GC-treated heart transplant recipients, in comparison with a 35% incidence of the placebo group (Sartori et al., 1999). Hypertension induced by therapeutic GCs is more prevalent in patients with high doses of GCs and occurs often in elderly patients with a positive family history of essential hypertension (Sholter and Armstrong, 2000). Cardiovascular actions of GC are also of physiological impact, because higher levels of the natural steroid cortisol, as seen in 80% of patients affected by Cushing’s syndrome, is dose dependently associated with increase of blood pressure (Kelly et al., 1998). Experimental data indicate that although cortisol-induced hypertension is characterized by Na\(^+\) retention and blood volume expansion, studies with synthetic steroids such as dexamethasone suggest that the hypertensive effect is to a substantial degree independent of the mineralocorticoid action (Saruta, 1996). Receptors for GC are present in endothelial and vascular smooth muscle cells (Provencher et al., 1995), and their activation increases response of the vasculature to catecholamines and angiotensin II (Walker and Edwards, 1994; Saruta, 1996). A recent study used genetically modified mice to demonstrate a functional link between dexamethasone-induced hypertension and reduced endothelial NO synthase activity (Wallenarth et al., 2004).

Blood pressure in humans is tightly controlled by several physiological systems interacting in a complex manner. Baroreceptors, natriuretic peptides, the renin-angiotensin-aldosterone system, the kinin-kallikrein system, the adrenergic receptor system, nitric oxides, and endothelin are all among them (Lifton et al., 2001). GC overuse can cause Na\(^+\) retention, hypokalemia, and hypertension by influencing these systems in different ways, thus causing severe heart problems (Schäcke et al., 2002). There is evidence that reduced NO production and consequently reduced plasma NO\(_2\)/NO\(_3\) (the oxidation products of NO) caused by a down-regulation of endothelial NO synthase contributes to the development of systemic cardiovascular side effects associated with high doses of GC (Li et al., 1992; Wallerath et al., 1999). Reduced NO bioavailability alters mainly vascular homeostasis by causing endothelial dysfunction and impairment of relaxing activity of smooth muscle cells, leading to hypertension and atherosclerosis, all of which are major cardiovascular complications in patients on long-term therapy with GCs (Iuchi et al., 2003). Conversely, therapeutic interventions aimed at improving NO bioavailability by releasing nitric oxide and nitrate species in biological fluids can be advantageous in preventing cardiovascular pathologies (for a recent review, see Cuzzocrea et al., 2001). Supported by these reasons, the present study was designed to compare the effects of prolonged treatment with a widely used GC such as prednisolone in relation to its NO-releasing nitro-steroid, or NCX-1015. NCX-1015 is emerging as a prototype of a new class of GC able to release NO species in biological fluids [see Baraldi et al., (2004) for electron spin resonance analyses] thus endowed with anti-inflammatory actions (Paul-Clark et al., 2000; Perreotti et al., 2003). In our hands, prednisolone administration to rats produced marked alterations in the cardiovascular system that followed both a time- and dose-dependent profile. At the highest dose tested of 6.9 µg/kg per day, prednisolone augmented MABP as early as after 1 week of treatment, and this was associated with significant alterations in +dP/dt and renal vascular resistance and blood flow. Pharmacologically relevant, the intermediate dose tested of 1.38 µmol/kg required a 3-week treatment to produce comparable alterations in MABP, RBF, and RVR. It was remarkable that NCX-1015 did not cause any of these changes. The importance of this negative set of experiments is obvious, not only in terms of potential development of GC with lower impact of side effects but also because the doses used are within the range found to possess anti-inflammatory and antiarthritic properties in the rat (Paul-Clark et al., 2002; Turesin et al., 2003) and mouse (Fiorucci et al., 2002; Paul-Clark et al., 2000). It was also important to confirm the efficacy of the doses used in the current experimental conditions, and we found that prednisolone and NCX-1015 caused

### Table 3

<table>
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<tr>
<th>Experimental Group</th>
<th>Basal</th>
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<th>Day 21</th>
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<tbody>
<tr>
<td>Vehicle</td>
<td>14.6 ± 2.0</td>
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<td>NCX-1015, 1.38</td>
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<td>16.4 ± 3.4</td>
<td>22 ± 3.0*</td>
<td>24 ± 2.7*</td>
</tr>
<tr>
<td>NCX-1015, 6.90</td>
<td>14.5 ± 2.4</td>
<td>22 ± 2.6*</td>
<td>28 ± 3.1**</td>
<td>33 ± 2.8**</td>
</tr>
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\*P < 0.05, **P < 0.01 significant versus proper vehicle group.

### Table 4

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Basal</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
</tr>
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<tr>
<td>Vehicle</td>
<td>67 ± 9</td>
<td>72 ± 11</td>
<td>64 ± 8</td>
<td>68 ± 12</td>
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<tr>
<td>Pred, 1.38</td>
<td>69 ± 10</td>
<td>64 ± 12</td>
<td>42 ± 19</td>
<td>38 ± 11*</td>
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<tr>
<td>Pred, 6.90</td>
<td>63 ± 13</td>
<td>42 ± 9*</td>
<td>29 ± 6**</td>
<td>18 ± 9**</td>
</tr>
<tr>
<td>NCX-1015, 1.38</td>
<td>67 ± 9</td>
<td>69 ± 10</td>
<td>39 ± 15</td>
<td>42 ± 8*</td>
</tr>
<tr>
<td>NCX-1015, 6.90</td>
<td>71 ± 14</td>
<td>39 ± 11*</td>
<td>31 ± 13*</td>
<td>21 ± 10**</td>
</tr>
</tbody>
</table>

\*P < 0.05, **P < 0.01 versus proper vehicle values.

---

**TABLE 4**

Effect of drug treatment on plasma corticosterone levels

Rats were treated for 3 weeks with vehicle (peanut oil; 0.5 ml/rat/day i.p.), prednisolone (Pred), or NCX-1015 (0.69, 1.38, or 6.90 µmol/kg/day i.p. for either steroid). Data are values of corticosterone (nanograms per milliliter) and are reported as mean ± S.E.M. of five rats per group.
similar degrees of suppression in circulating corticosterone levels.

In the second part of the study, we sought to provide at least a partial molecular explanation to the hypertensive effect of prednisolone. Because GC increases the transcription of the preproendothelin-1 gene (Provencher et al., 1998) as well as the expression of ET-1 receptors (Villeneuve et al., 2000), we measured plasma levels of this peptide. In line with MABP and other cardiovascular measurements, prednisolone but not NCX-1015 increased ET-1 plasma levels in a time- and dose-dependent manner. Regression analysis demonstrated a significant association between plasma ET-1 and changes in MABP or RVR. Therefore, in the second set of experiments, we tested the effects of three distinct types of ET-1 receptor antagonists. Minor changes (~10 mm Hg) in MABP and other parameters were measured after treatment with the ET_{A} receptor antagonist BQ-788, the ET_{A} receptor antagonist FR139317, or the mixed ET_{A}/ET_{B} receptor antagonist SB209670. This is in line with the modest, and sometimes conflicting, results published with ET-1 antagonists in experimental models of hypertension. As an example, the combined ET_{A}/ET_{B} receptor antagonist bosentan produced minor changes on elevated MABP and vascular hypertrophy in deoxycorticosterone acetate-salt hypertensive rats (Li et al., 1994), whereas it did not modify MABP in spontaneously hypertensive rats (Li and Schiffrin, 1995). Administration of another orally active ET_{A} receptor antagonist showed a similar discrepancy of effect between the two models (Bird et al., 1995). Thus, ET_{A} receptor antagonism was active in abrogating the marked cardiovascular alterations produced by prednisolone.

It is likely that the lack of effect of NCX-1015 on the cardiovascular parameters measured in this study may be due to the release of NO or NO species, from the linker attached to the steroid moiety of the molecule. Indeed, NCX-1015 treatment increased plasma nitrate/nitrite levels in a time-dependent manner. NCX-1015 releases NO species in human plasma; this is possibly supported by endogenous esterases and occurs also after administration in the experimental animal (Paul-Clark et al., 2000). Recent electron spin resonance analysis confirmed NCX-1015 ability to release

**Fig. 5.** Correlation between plasma ET-1 levels and cardiovascular markers. A, reanalysis of the data presented in Figs. 1A and 4A, demonstrating a positive relationship between plasma ET-1 levels and MABP. B, as in A, showing the relationship between plasma ET-1 and RVR, reported in Fig. 3B.

**Fig. 6.** Effect of ET-1 receptor antagonists of the hypertensive effect of prednisolone. A, rats were treated for 3 weeks with vehicle (peanut oil; 0.5 ml/rat/day), prednisolone (Pred; 6.9 μmol/kg/day) alone or supplemented with FR139317 (FR139; 30 mg/kg/day), SB209670 (SB209; 10 mg/kg/day), or BQ-788 (1 mg/kg/day) in the drinking water. At weekly intervals, MABP was measured by tail cuff as described under Materials and Methods. B, as in A, but the antagonists were administered in the absence of Pred. Data are mean ± S.E.M. of six rats per group. *, P < 0.05 and **, P < 0.01 versus respective vehicle value; *, P < 0.05 and **, P < 0.01 versus corresponding vehicle value; and #, P < 0.05 versus respective Pred group.
In conclusion, the present study attempted to provide experimental mechanistic support to the well known cardiovascular effects associated with long-term GC use, and it is the first to demonstrate an involvement of the ET-1 system in the development of prednisolone-induced hypertension. These results may also have a dual impact on the development of new therapeutics: on one hand, the further development of NCX-1015 and related nitro-steroids; and on the other hand, the potential application of ET\textsubscript{A} antagonists in association with long-term GC administration.

References


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