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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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Running Title: Nitro-prednisolone does not cause hypertension

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List of non standard abbreviations: BQ-788, (N-cis-2,6-dimethylpiperidinocarbonyl-L-γ-metLeu-d-L-methoxy-carbonylTrp-D-Nle); ET, endothelin; ET_A/ET_B, ET receptor type A or B; FR139317, ((+)-(1S,2R,3S)-3-(2-carboxymethoxy-4-methoxyphenyl)-1-(3,4methylenedioxyphenyl)-5-(prop-1-yloxy)indane-2-carboxylic acid); GC, glucocorticoid; MABP, mean arterial blood pressure; NCX-1015 (prednisolone 21-[4'-nitrooxymethyl]benzoate); NO, nitric oxide; RBF, renal blood flow; RVR, renal vascular resistance; SB209670, ((R-2-[(R)-2-[(S)-2-[[1-(hexahydro-1H-azepinyl)]-carbonyl]amino-4-methylpentanoyl]-amino-3-(2-pyridil)propionic).

Section: Cardiovascular

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 one week for the dose of 6.9 μmol/kg i.p. (n=10, $P<0.05$), and three weeks for the lower dose of 1.38 μmol/kg. A similar dichotomy of behaviour 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 i.p.) and time-dependent (1-3 weeks) fashion. Similar profiles were obtained for plasma nitrate values, though they were increased selectively following 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 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 i) a lack of occurrence of cardiovascular alterations by nitro-releasing derivative of prednisolone (NCX-1015), and ii) a functional link between prednisolone effects and the endogenous endothelin-1 system.

1. Introduction

Glucocorticoids (GC) 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 (Saruta, 1996; Whitworth, 1994). Marked side effects include obesity with the classical "moon face", hirsutism, cataract, osteoporosis, diabetes mellitus, immune-suppression and cardiovascular disorders including hypertension and atherosclerosis (Ross et al., 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 GC (Ross et al., 1982). Plasma volume expansion due to sodium retention gives a minor contribution to GC cardiovascular effects (Saruta, 1996; Whitworth et al., 1989; Whitworth, 1994). 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 following excess use of GC (Pirpiris et al., 1992; Saruta, 1996; Whitworth, 1994). The molecular mechanism whereby GC excess causes the increase in vascular resistance and hypertension has not been fully elucidated, though 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 (Luscher et al., 1997; Weber et al., 1998). Among these factors a major function is played by prostanoids 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 et al., 1997).

Vascular resistance, blood pressure and cardiac function are also increased following activation of the endothelin system (Yanagisawa et al., 1988; Ishikawa et al., 1988). Endothelin 1 (ET-1) is a 21 amino acid long potent vaso-active peptide (Provencher et al., 1998). This peptide acts in strict cooperation, and in an antagonistic fashion, with the NO system to produce a fine regulation of the degree of vessel dilation/contraction (Schini et al., 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 et al., 1991; Kojda et al., 1997; Rothermund et al., 2000). Some of these pathological conditions are indeed characterised by an augmented ET-1 synthesis or release (Schiffrin et al., 1997; Ruschitzka 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 over-expressed in the vasculature has been reported (reviewed by Iglarz and Schiffrin, 2003). The same applies in the clinical settings, since patients affected by moderate to severe hypertension present increased vascular endothelial and smooth muscle cells levels of prepro-ET-1 mRNA (Iglarz and Schiffrin, 2003).

Since GC are the most potent anti-inflammatory agents available to date, their therapeutic use would greatly 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'-nitrooxymethyl]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 pro-inflammatory enzymes (Paul-

Clark et al., 2000). A subsequent study demonstrated a lack of osteoclast stimulating effect by NCX-1015, and this appeared 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: on one hand, 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, on the other hand, test if 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.

2. Materials and methods

2.1. Materials

NCX-1015 (prednisolone 21-[4'-nitrooxymethyl]benzoate) was synthesised at NicOx Research Institute (Milan, Italy) as already described (Paul-Clark et al., 2000). Prednisolone was supplied by NicOx, while FR 139317 ((R-2-[(R)-2-[(S)-2-[[1-(hexahydro-1H-azepinyl)]-carbonyl]amino-4-methylpentanoyl]-amino-3-(2-pyridil)propionic) was from Parke Davis Pharmaceutical Research (Ann Arbor, Michigan, USA), SB209670 ((+)-(1S,2R,3S)-3-(2-carboxymethoxy-4-methoxyphenyl)-1-(3,4 methylenedioxyphenyl)-5-(prop-1-yloxy)indane-2-carboxylic acid) was from GlaxoSmithKline (UK) and BQ-788 (N-cis-2,6-dimethylpiperidinocarbonyl-L-γ-metLeu-d-L-methoxy-carbonylTrp-D-Nle) (from Banyu Pharmaceutical Co. (Tokyo, Japan).

2.2. Animals and drug treatment

Male Sprague-Dawley rats (body weight ~150 g at the beginning of experimentation) were purchased from Charles River (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 EEC regulations (O.J. of E.C. L 358/1 12/18/1986).

A total number of 118 rats were used in the study. Following recording of basal values (see below), animals were treated daily with a single dose of GC given intraperitoneally (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 prednisolone 6.9 μmol/kg 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 (Moreau et al., 1997). The average intakes of the ET-1 antagonists were: 30 mg/kg/day for the selective type A (ET_A)receptor antagonist FR139317 (Sogabe et al., 1993); 1 mg/kg/day for the selective ET-1 type B (ET_B, Ishikawa et al., 1994) receptor antagonist BQ-788; 10 mg/kg/day for the non-selective ET_A/ET_B antagonist (Ohlstein et al., 1994) SB209670. These doses are in line with those used in other studies addressing the effect of chronic treatment (Kohzuki et al., 1998; Fujihara et al., 1995; 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 following mild halothane anesthesia at the end of each week of treatment.

2.3 Determination of systolic blood pressure

Mean arterial blood pressure (MABP) was determined every week using a non-invasive methodology (tail cuff) as described (Bhanot et al., 1994; Galipeau et al., 2001). Briefly, rats were warmed in a heater for single holder LE 5610 **(**Letica, 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 a LE5200 apparatus **(**Letica, Barcelona, Spain). 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).

2.4 Determination of cardiac function

At the end of the treatment period (week 3 or 21 days), rats were anesthetised 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 microtip pressure transducer catheter (model SPC-320, Millar Instruments, Sakai et al., 1996) inserted into the right carotid artery. +dP/dt was derived by active analogue differentiation of the pressure signal by means of a Mac Lab system (AD Instrument, Hastings, UK). Heart rate was monitored by electrocardiogram recording through a Hellige cardiotest EK41 (Hellige, Freiburg, Germany).

2.5 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 flow meter (Transonic Systems Inc, New York, USA) as described by D'Amico at al. (1996). Renal vascular resistance (RVR) was then calculated as the ratio MABP/RBF.

2.6 Biochemical Analyses

After the completion of the hemodynamic measurements a blood sample was taken. Aliquots of anti-coagulated blood (5% EDTA) were centrifuged at 300 *g* for 15 min. Supernatant (plasma) samples were stored at –20°C prior to analysis. Plasma ET-1 levels were quantified using a specific enzyme immunoassay (Cayman Chemical, Ann Arbor, Michigan USA), which shows high sensitivity (detection limit 1.5 pg/ml; data furnished by the manufacturer): unknown values in plasma samples were compared to a standard curve constructed with 0-250 pg/ml rat ET-1. Plasma 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). Corticosterone was measured with an ELISA kit (IDS, Boldon, U.K.) with a detection limit of 3.0 ng/ml.

2.7 Statistics

Data are reported as mean ± SEM of 10 rats per group. Differences amongst experimental groups were analysed by analysis of variance (ANOVA). An F value corresponding to a probability less than <0.05 was required to reject the null hypothesis, inter-group variations were determined by the Dunnett's test (comparison *vs.* the vehicle-treated group) or the Bonferroni's test (comparison between appropriate prednisolone and NCX-1015 groups). In either case a P value <0.05 was taken as significant.

3. Results

3.1 Effect on body weight

No significant changes in body weights were recorded across the treated experimental groups, when compared either amongst themselves or with vehicle group (Table 1).

3.2. Effects on mean arterial blood pressure (MABP)

Basal MABP values around 110 mm Hg were measured in all animals prior to beginning of treatment. These values did not significantly change after i.p. administration of vehicle over the three week period (Figure 1A). In contrast, treatment of rats with prednisolone caused significant alterations in MABP both in a dose- and time-dependent fashion. 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 max of 150 ± 1.7 mmHg at week 3 (Figure 1A). At the intermediate prednisolone dose of 1.38 μmol/kg (corresponding to 0.5 mg/kg), three-week, but not one- or two-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) (Figure 1A).

Importantly, equimolar doses of NCX-1015 failed to cause any significant changes in MABP from the vehicle treated group (Figure 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 week 2 and week 3; n=10 rats).

3.3 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 ($P<0.01$ *vs.* 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 ($P<0.01$ vs. equimolar dose of prednisolone; not significant vs. vehicle). Neither GC or dose treatment affected the heart rate values, when compared to vehicle treated control animals (Table 2).

3.4 Effects 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 to vehicle-treated rats (Figure 3A and 3B). In contrast, no changes in either RVR or RBF were observed following 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.

3.5 Biochemical measurements in rat plasma samples

Basal ET-1 immuno-reactivity was detected in the plasma of vehicle-treated rats (Figure 4A and 4B). Prednisolone (1.38 μmol/kg or 0.5 mg/kg), administered for three weeks, significantly ($P<0.05$) increased plasma ET-1 levels (Figure 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 one week (Figure 4B). At this dose, prednisolone produced almost a three-fold increase in ET-1 levels ($P<0.01$) compared to vehicle-treated rats, as measured at the three-week time-point (Figure 4B). The lower dose tested of 0.69 μmol/kg i.p. (0.25 mg/kg) prednisolone did no modify plasma ET-1 contents at any time (data not shown). Noteworthy, administration of equivalent doses of NCX-1015 to rats for three weeks did not produce any significant alterations in plasma ET-1 levels (Figure 4A and 4B).

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 three weeks mildly decreased circulating levels of both either 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 (Table 3 and Table 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 measurements, also these parameters changed in a dose- and time-dependent fashion.

3.6 Functional relationship between ET-1 and cardiovascular changes

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

3.7 Endothelin receptor antagonists: effect on prednisolone-induced cardiovascular alterations

Administration in the drinking water of FR139317 (30 mg/kg/day) to the rats greatly attenuated the increase in MABP induced by daily administration of prednisolone (6.9 μmol/kg) (Figure 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) (Figure 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 (Figure 6A). Given alone, the antagonists produced modest changes in MABP. Notably, FR139137 reduced MABP by ~10 mmHg, whereas BQ-788 increased it by a similar degree (Figure 6B).

The ET_A antagonist FR139137 was effective also in reducing prednisolone-induced increase in +dP/dt (Figure 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 (Figure 6) were reflected in minimal alterations in +dP/dt (Figure 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 analysed. In analogy to what reported above (Figure 3), 3-week treatment with prednisolone produced significant increases in RBF and RVR (Figure 7B and 7C): the selective ET_A antagonist was active in preventing them, with SB209670 being equally effective, whereas BQ718 produced a modest augmentation of GC action (Figure 7B and 7C).

4. Discussion

In this study we reproduced some of the cardiovascular alterations known to characterise 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, 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 (Sholter and Armstrong, 2000: Sartori et al., 1999). 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 ct al., 1998). On the same wave, a hypofibrinolytic state due to increased plasminogen activator inhibitor-1 activity was reported in 69% of the GC-treated heart transplant recipients, in comparison to 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 Aemstrong, 2000). Cardiovascular actions of GC are also of physiological impact, since 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 el 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 interesting study used genetically modified mice to demonstrate a functional link between dexamethasone-induced hypertension and reduced endothelial NO synthase activity (Wallerath et al., 2004).

Blood pressure in humans is tightly controlled by several physiologic systems interacting in a complex fashion. Baro-receptors, 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, 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 GC (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; Perretti et al., 2003). In our hands, prednisolone administration to rats produced marked alterations in the cardio-vascular 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 one 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 three-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 anti-arthritic properties in the rat (Paul-Clark et al., 2000; Turesin et al., 2003) and mouse (Paul-Clark et al., 2002; Fiorucci et al., 2002). 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 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. Since GC increase 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 mmHg) in MABP and other parameters were measured following treatment with the ET_B receptor 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 et al., 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 fashion. 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 nitrate species (Baraldi et al., 2004). Relevantly, the impact of NO species released from NCX-1015 becomes of particular relevance following longer *in vitro* incubations (Paul-Clark et al., 2002) or prolonged treatment (Perretti et al., 2003) as in the present study. NCX-1015-derived NO would likely be mechanistically linked to the lack of effect of this steroid on ET-1 plasma concentrations. NO, in fact, inhibits synthesis and release of ET-1 from endothelial cells (Brunner et al., 1995). Furthermore, the patho-physiological cardiovascular actions of ET-1 have long been known to be quenched by NO donors (Boulanger et al., 1990; Gardiner et al., 1990; Chyu et al., 1992; D'Amico et al., 1994).

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 side the further development of NCX-1015 and related nitro-steroids; on the other hand, the potential application of ET_A antagonists in association with long-term GC administration.

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Footnotes

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Legends to the Figures

Figure 1. Effect of drug treatment on mean arterial blood pressure. (A) Rats were treated daily with vehicle (peanut oil, 0.5 ml per rat i.p.) or prednisolone (Pred, 0.69, 1.38 or 6.90 μmol/kg i.p.) and mean arterial blood pressure (MABP) measured by tail cuff as described in the *Methods* section at weekly intervals. (B) as in panel A, but NCX-1015 (NCX, 0.69, 1.38 or 6.90 μmol/kg i.p.) was given instead of Pred. Data are mean ± SEM of 10 rats per group. *P<0.05 and **P<0.01 *vs.* respective vehicle value.

Figure 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 per 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 (mmHg per sec) was measured as described in the *Methods* section. Data are mean ± SEM of 10 animals per group. * P<0.05 and ** P<0.01 *vs.* vehicle group, and # P<0.05 *vs.* respective Pred group.

Figure 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 per 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 renal blood flow (RBF) was measured as described in the *Methods* section. (B) as in panel A, but renal vascular resistance (RVR) is reported. In either panel, data are mean ± SEM of 10 rats per group. * P<0.05 and ** P<0.01 *vs.* vehicle group, and # P<0.05 *vs.* respective Pred group.

Figure 4. Distinct effect of prednisolone and NCX-1015 on plasma ET-1 levels. (A) Rats were treated daily with vehicle (peanut oil, 0.5 ml per rat i.p.), prednisolone (Pred, 1.38 μmol/kg i.p.) or NCX-1015 (NCX, 1.38 μmol/kg i.p.), and blood collected at weekly interval for determination of ET-1 content. (B) as in panel A, but steroid doses were 6.9 were μmol/kg i.p. per day. Time 0

refers to vehicle group at 3 weeks, though values were identical also for week 1 and week 2 (not shown). In either panel, data are mean ± SEM of 10 rats per group. * P<0.05 and ** P<0.01 *vs.* vehicle group, and # P<0.05 *vs*. respective Pred group.

Figure 5. Correlation between plasma ET-1 levels and cardiovascular markers. (A) Re-analysis of the data presented in Figure 1A and 4A, demonstrating a positive relationship between plasma ET-1 levels and mean arterial blood pressure (MABP). (B) as in panel A, showing the relationship between plasma ET-1 and renal vascular resistance (RVR), reported in Figure 3B.

Figure 6. **Effect of ET-1 receptor antagonists of the hypertensive effect of prednisolone.** (A) Rats were treated for three 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, mean arterial blood pressure (MABP) was measured by tail cuff as described in the *Methods* section. (B) as in panel A, but the antagonists were administered in the absence of Pred. Data are mean ± SEM of 6 rats per group. * P<0.05 and ** P<0.01 *vs*. respective vehicle value. * P<0.05 and ** P<0.01 *vs.* corresponding vehicle value, and # P<0.05 *vs.* respective Pred group.

Figure 7. Effect of endothelin receptor antagonists of the extent of cardiac +dP/dt, renal blood flow and vascular resistance. (A) Rats were treated for three weeks with vehicle (peanut oil, 0.5 ml/rat/day) or prednisolone (Pred; 6.9 μmol/kg/day) and had access to normal drinking water or supplemented with FR139317 (FR139; 30 mg/kg/day), SB209670 (SB209; 10 mg/kg/day) or BQ-788 (1 mg/kg/day). After 3 weeks, +dP/dT (mmHg per sec) was measured as described in the *Methods* section. (B) and (C) as in panel A, but renal blood flow (RBF) or renal vascular resistance (RVR) were determined. In all panels, data are mean ± SEM of 6 rats per group. * P<0.05 and ** P<0.01 *vs.* respective water/vehicle value whereas # P<0.05 *vs.* respective water/Pred group.

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Table 1. Effects of drug treatment on body weight.

Experimental Groups	Basal	Day 7	Day 14	Day 21
Vehicle	189 ± 3	191 ± 3	207 ± 3	223 ± 3
Pred 0.69	188 ± 3	195 ± 2	209 ± 3	220 ± 4
Pred 1.38	186 ± 2	195 ± 3	207 ± 2	221 ± 4
Pred 6.90	195 ± 2	194 ± 3	208 ± 3	224 ± 3
NCX-1015 0.69	186 ± 2	197 ± 4	203 ± 3	228 ± 2
NCX-1015 1.38	190 ± 4	196 ± 2	202 ± 3	225 ± 3
NCX-1015 6.90	191 ± 2	197 ± 2	206 ± 3	224 ± 2

Rats were treated for three 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 (g) and are reported as mean ± SEM of 10 rats per group.

Table 2. Effects of drug treatments on heart rate.

Experimental Groups	Heart Rate (beats per min)
Vehicle	368 ± 9
Pred 0.69	361 ± 7
Pred 1.38	382 ± 6
Pred 6.90	362 ± 8
NCX-1015 0.69	373 ± 6
NCX-1015 1.38	373 ± 7
NCX-1015 6.90	378 ± 7

Rats were treated for three 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 heart rate (beats x min^{-1}) and are reported as mean ± SEM of 10 rats per group.

Table 3. Effect of drug treatment on plasma nitrate/nitrite levels.

Experimental Groups	Basal	Day 7	Day 14	Day 21
Vehicle	14.6 ± 2.0	14.0 ± 1.9	14.3 ± 2.0	14.2 ± 2.2
Pre 0.69	14.9 ± 3.0	14.5 ± 2.6	14.8 ± 1.8	14.0 ± 2.2
Pred 1.38	14.4 ± 2.7	14.2 ± 2.1	12.6 ± 1.9	9.1 ± 1.3*
Pred 6.90	13.8 ± 2.1	8.9 ± 1.8*	9.0 ± 2.1*	8.5 ± 1.9*
NCX-1015 0.69	14.2 ± 1.9	14.1 ± 2.4	14.6 ± 2.6	14.9 ± 1.9
NCX-1015 1.38	14.2 ± 3	16.4 ± 3.4	22 ± 3.0*	24 ± 2.7*
NCX-1015 6.90	14.5 ± 2.4	22 ± 2.6*	28 ± 3.1**	33 ± 2.8**

Rats were treated for three 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 nitrate/nitrite (μmol/L) and are reported as mean ± SEM of 5 rats per group. * $P<0.05$, **$P<0.01$ significant *vs.* proper vehicle group.

Table 4. Effect of drug treatment on plasma corticosterone levels.

Experimental Groups	Basal	Day 7	Day 14	Day 21
Vehicle	67 ± 9	72 ± 11	64 ± 8	68 ± 12
Pred 1.38	69 ± 10	64 ± 12	42 ± 19	38 ± 11*
Pred 6.90	63 ± 13	42 ± 9*	29 ± 6**	18 ± 9**
NCX-1015 1.38	67 ± 9	69 ± 10	39 ± 15	42 ± 8*
NCX-1015 6.90	71 ± 14	39 ± 11*	31 ± 13*	21 ± 10**

Rats were treated for three weeks with vehicle (peanut oil, 0.5 ml/rat/day i.p.), prednisolone or NCX-1015 (1.38 or 6.90 μmol/kg/day i.p. for either GC). Data are values of corticosterone (ng/ml) and are reported as mean ± SEM of 5 rats per group. * $P<0.05$, ** $P<0.01$ *vs.* proper vehicle values.

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Table 5. Values of heart rate as measured after a 21-day treatment with ET-1 antagonists.

Experimental group	Heart Rate (beats per min)
Vehicle	374 ± 6
Prednisolone	367 ± 9
Prednisolone + FR139317	374 ± 9
Prednisolone + SB209670	368 ± 9
Prednisolone + BQ-788	363 ± 8
FR139317	360 ± 7
SB209670	368 ± 8
BQ-788	369 ± 9

Rats treated for three weeks with vehicle (peanut oil, 0.5 ml/rat/day), prednisolone (6.9 μmol/kg/day) alone or supplemented with FR139317 (30 mg/kg/day), SB209670 (10 mg/kg/day) or BQ-788 (1 mg/kg/day) in the drinking water. Each value represents the mean ± SEM of 6 rats per group.

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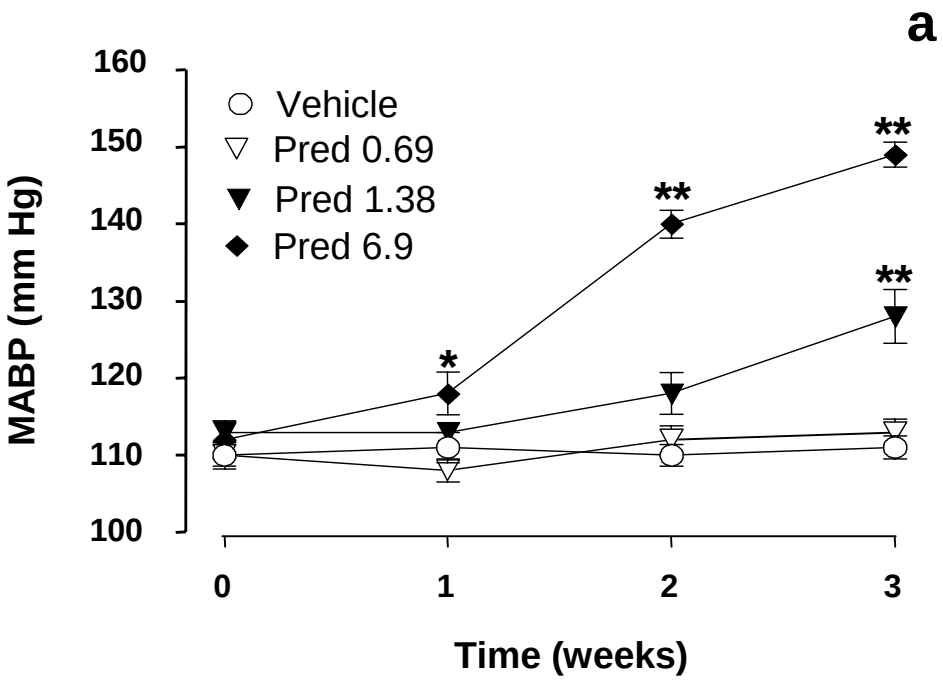

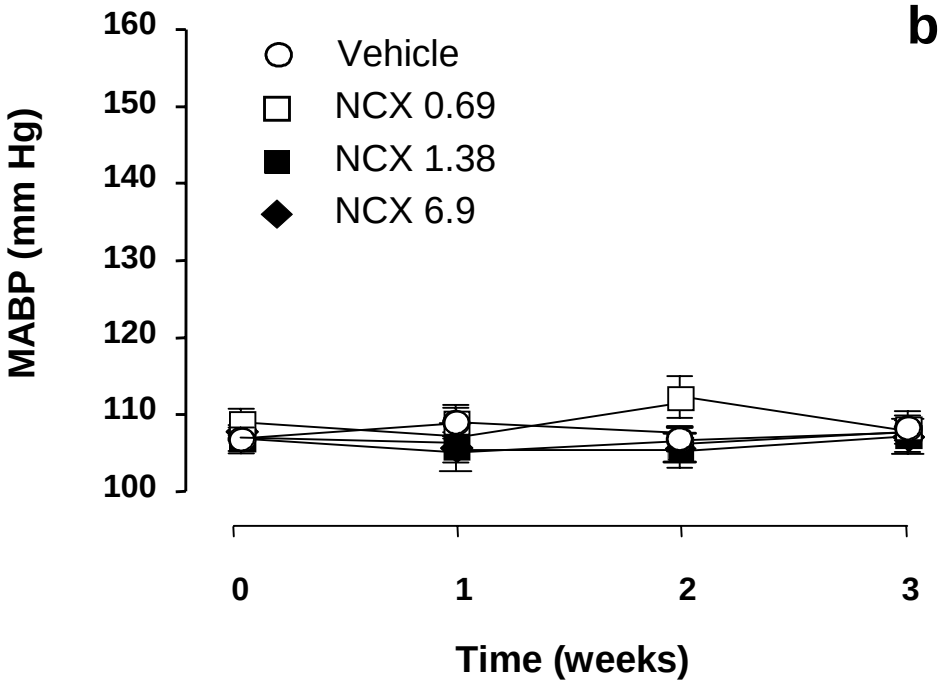

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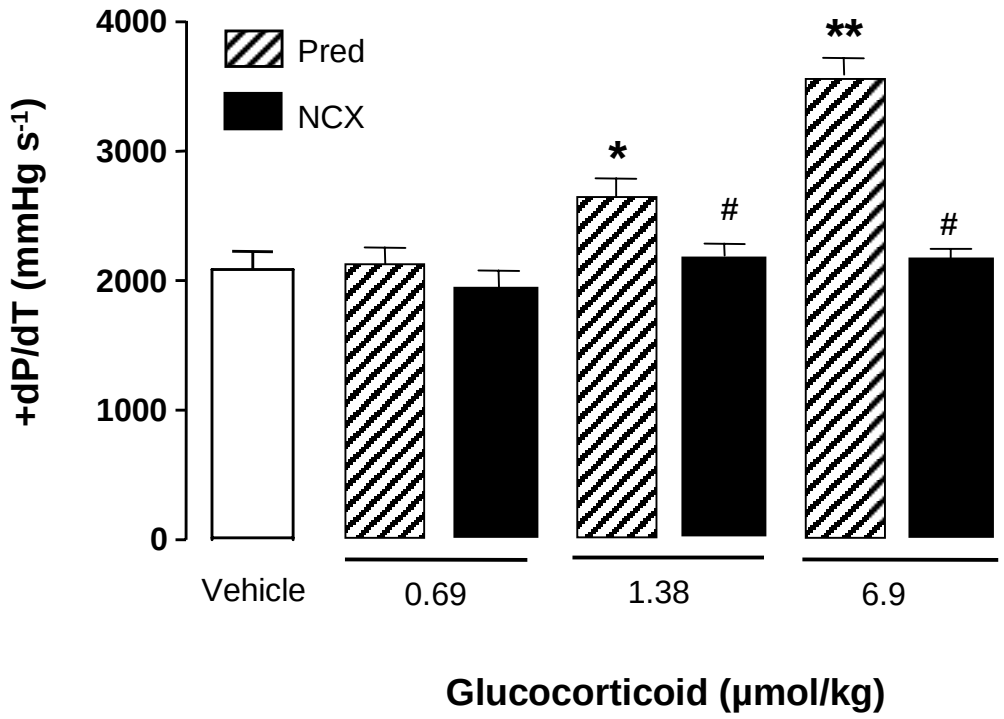

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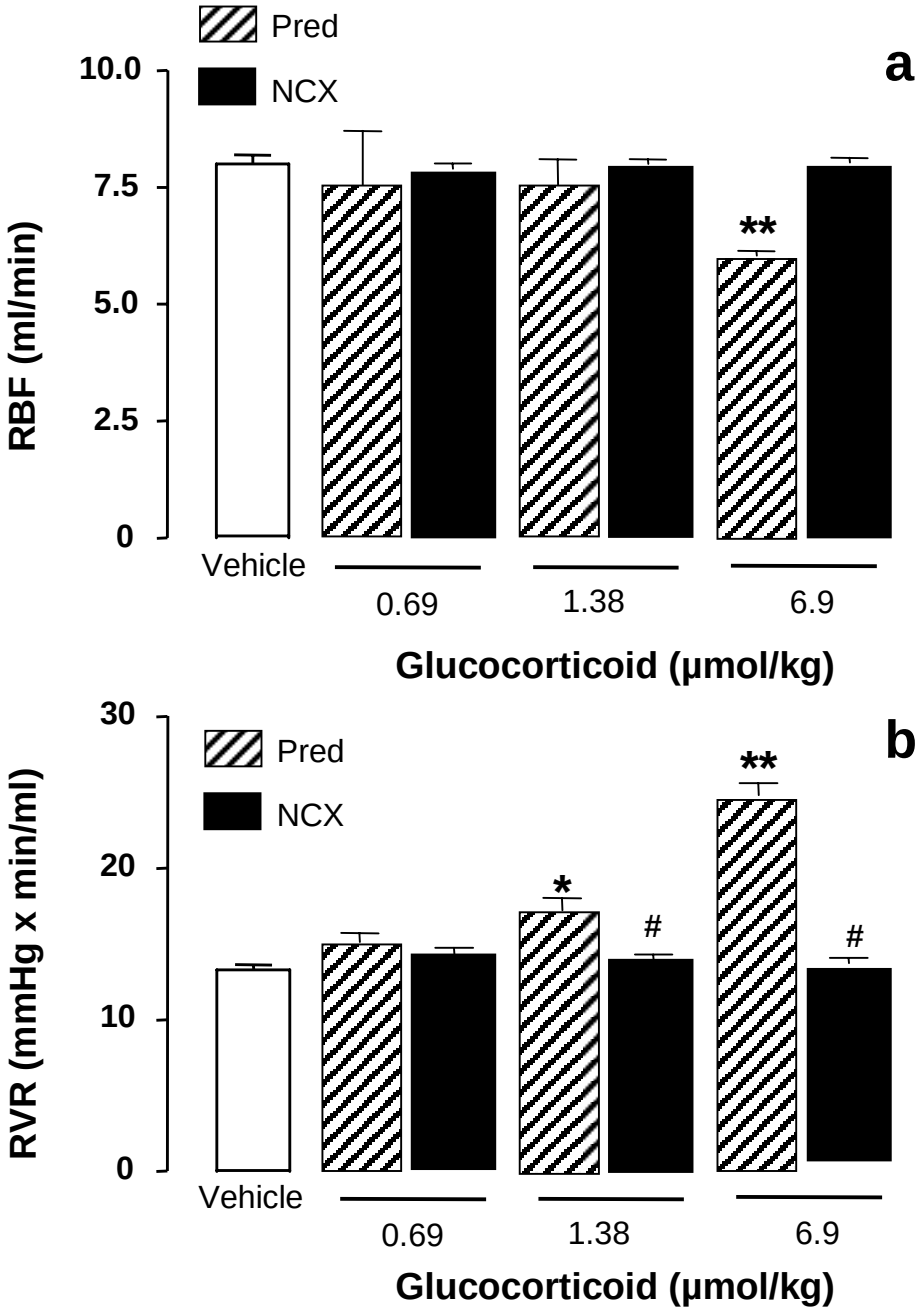

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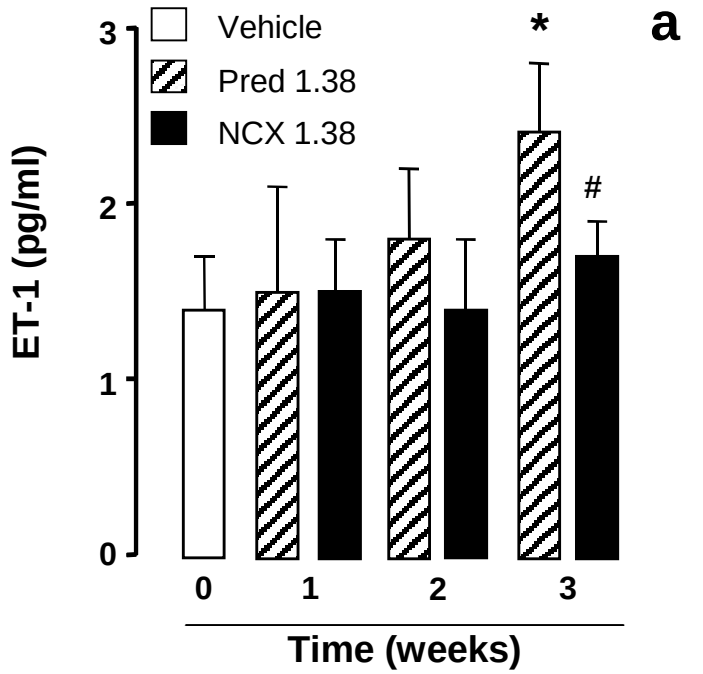

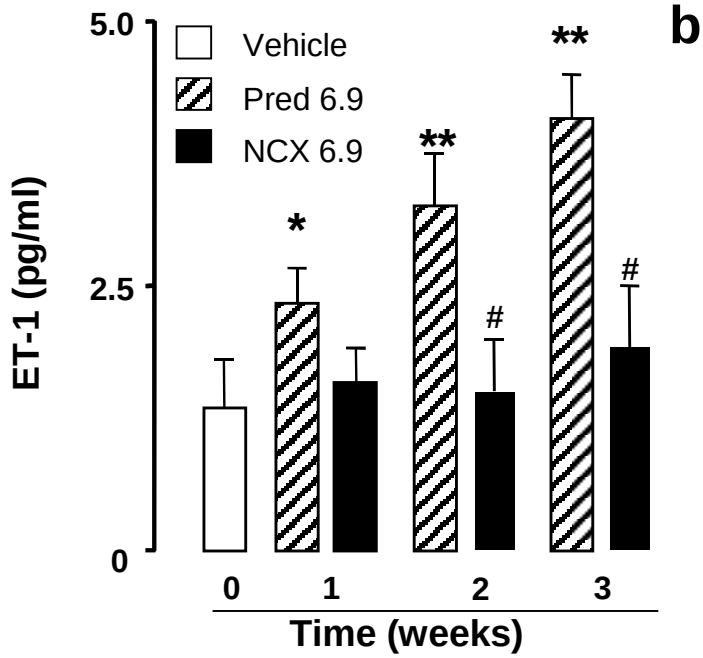

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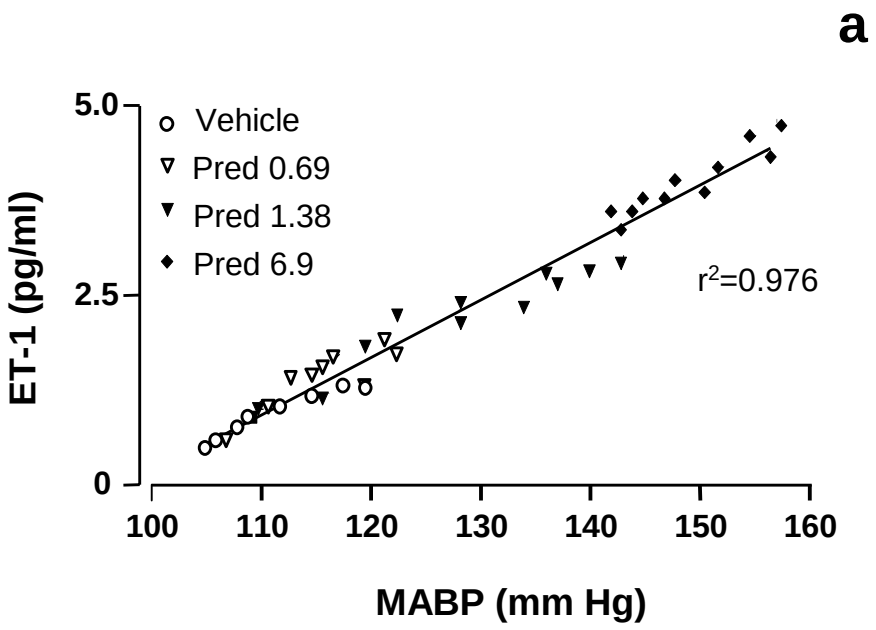

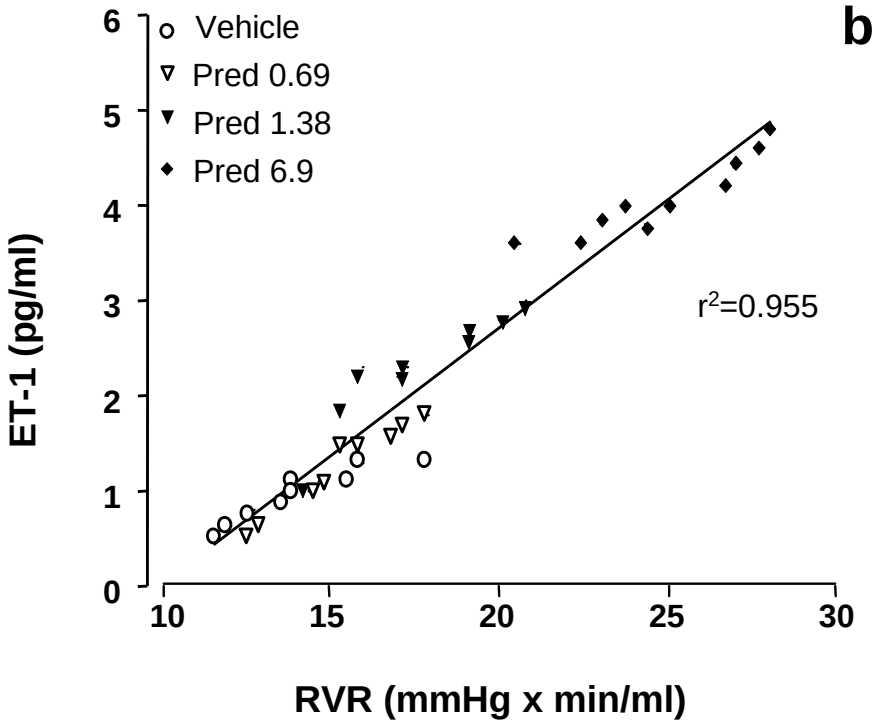

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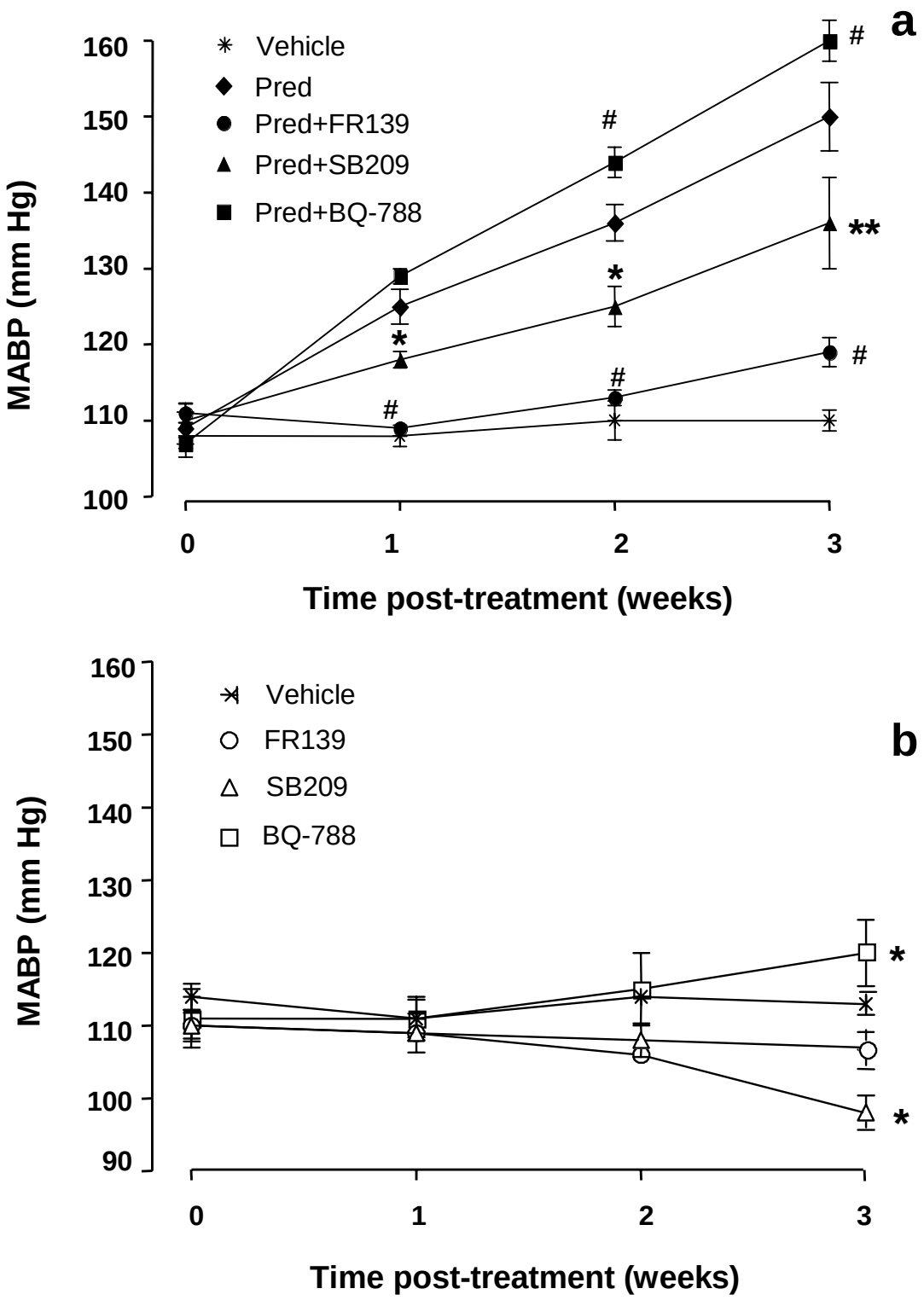

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