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Effects of Chromium Picolinate on Vascular Reactivity and Cardiac Ischemia Reperfusion Injury in Spontaneously Hypertensive Rats

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Abstract

Chromium picolinate [Cr(pic)3] is a nutritional supplement widely promoted to exert beneficial metabolic effects in patients with type 2 diabetes/impaired glucose tolerance. Frequent comorbidities in these individuals include systemic hypertension, abnormal vascular function and ischemic heart disease but information on effects of the supplement on these aspects is sparse. Utilizing male spontaneously hypertensive rats (SHR), we examined potential impact of Cr(pic)3 on blood pressure, vascular reactivity and myocardial ischemia reperfusion injury (IRI). Dietary Cr(pic)3 supplementation (as 10 mg chromium/kg diet for 6 weeks) did not affect blood pressure of the SHR. Also, neither norepinephrine (NE) and potassium chloride (KCl)-induced contractility nor sodium nitroprusside (SNP)-induced relaxation of aortic smooth muscle from the SHR was altered by Cr(pic)3 treatment. However, Cr(pic)3 augmented endothelium-dependent relaxation of aortas, produced by acetylcholine (ACh), and this effect was abolished by N-nitro-L-arginine methyl ester (L-NAME) suggesting induction of nitric oxide (NO) production/release. Treatment with Cr(pic)3 did not affect baseline coronary flow rate and rate-pressure-product (RPP) or infarct size following regional IRI. Nonetheless, Cr(pic)3 treatment was associated with improved coronary flow and recovery of myocardial contractility and relaxation following ischemia reperfusion insult. In conclusion, dietary Cr(pic)3 treatment of SHR neither alters blood pressure nor vascular smooth muscle reactivity, but causes enhancement of endothelium-dependent vasorelaxation associated with NO production/release. Additionally, while the treatment does not affect infarct size, it improves functional recovery of the viable portion of the myocardium following IRI.

Key words/phrases

Chromium picolinate; spontaneously hypertensive rat; vascular reactivity; heart; ischemia reperfusion injury

Introduction

Chromium, as a trivalent element, is considered an essential trace mineral for optimum health. The relatively better absorbed form of trivalent chromium, chromium picolinate [Cr(pic)3], is widely promoted as a dietary supplement for adjunctive therapy for type 2 diabetes mellitus/glucose intolerance [6, 8, 12, 15, 18, 20]. Among other effects, it has been

shown to improve glucose homeostasis by enhancing insulin sensitivity both in humans and animal disease models [6, 8].

While Cr(pic)3 has been extensively investigated in relation to carbohydrate metabolism, there are only a few studies devoted to its effects on the cardiovascular system [14, 17, 19, 24–27, 29]. The consideration of the cardiovascular effects of the supplement becomes more relevant in view of the documented association between type 2 diabetes/glucose intolerance and cardiovascular disorders such as abnormal vascular function, increased blood pressure and augmented susceptibility to ischemic heart disease [3, 4, 11, 28]. In this regard, it has been demonstrated that Cr(pic)3 treatment attenuates sucrose-induced exacerbation of blood pressure in the spontaneously hypertensive rats (SHR) in association with improvement in acetylcholine- and nitroprusside-induced vasodilatory responses [17, 25–26]. On the other hand, utilizing the insulin resistant JCR-LA-cp rat, Proctor et al. [27] showed reduced phenylephrine-induced contractile responses of endothelium-intact aortas from Cr(pic)3-supplemented rats although blood pressure was not reported. With respect to the myocardium, improvements in recovery from myocardial ischemia reperfusion injury (IRI) have been reported for an energy formula containing chromium-niacin as well as chromium-niacin per se [24, 29]; however, the potential effect of Cr(pic)3 on myocardial IRI and the mechanisms involved in its vascular action remain to be established. Thus the objective of this study was to determine the effects of oral Cr(pic)3 supplementation on blood pressure, vascular reactivity and the outcome of myocardial IRI in the same animal model of hypertension. For these studies, we used SHR that display a myriad of abnormalities of the cardiovascular system including marked elevation in blood pressure in association with other abnormal cardiovascular function [10]. Accordingly, we tested the hypotheses that dietary Cr(pic)3 a) attenuates hypertension in SHR and this effect is associated with reduced vascular smooth muscle contractility and enhancement of endothelium-dependent and independent vasorelaxation, and b) decreases susceptibility of the myocardium to IRI.

Methods and materials

Animals

Five-week-old male SHR were obtained from Harlan Laboratories (Indianapolis, IN). The animals were maintained at constant humidity ($60\pm 5\%$), temperature ($24\pm 1^\circ\text{C}$) and light cycle (6:00 AM–6:00 PM). All animals had free access to food and water. One week after arrival (i.e., at 6 weeks of age), the rats were randomly divided into two groups with $n=6$ /group. This sample size was used based on our previous experience and studies performed by other investigators regarding the cardiovascular system [2–4, 17, 25–26]. The animals in one group were given standard rat diet (Harlan Teklad diet # 8604) while those in the other group were switched to the chromium picolinate (Cr(pic)3)-enriched diet containing 10 mg/kg of chromium (Harlan Teklad diet # 07603); diets containing similar amounts of Cr(pic)3 have been used in previous studies [5, 7, 15, 20]. The ingredients contained in the standard diet are available on the website of Harlan Teklad (<http://www.harlan.com>). Treatment with Cr(pic)3 was initiated at 6 weeks of age and continued to 12 weeks of age. This interval was deemed appropriate because during this time period, SHR experience marked elevation in blood pressure [23]. Accordingly, we conjectured that Cr(pic)3 during this critical time interval would prevent/attenuate blood pressure in SHR thereby implicating a role for the nutritional supplement in modulation of regulatory mechanisms that elevate blood in SHR. Tail-cuff hemodynamics were measured 3 and 6 weeks after initiation of dietary regimens [20]. The animals were sacrificed after 6 weeks of (Cr(pic)3) supplementation; the heart was quickly removed and secured on the heart perfusion apparatus and the aortas were obtained for vascular reactivity studies. The use of animals for this study was in accordance with institutional guidelines for care and use of laboratory animals, based on the NIH guidelines. The protocol was approved by the Institutional Animal Care and Use Committee.

Vascular preparation and reactivity assessment

The isolated aortas from both groups of animals were carefully cleaned of fat and connective tissue and then cut into rings of 3–5mm of length. In some of the rings, the endothelium was removed mechanically by inserting the tip of a pair of forceps into the lumen of each ring and rotating it gently [2–4]. The endothelium of the rest of the rings was left undenuded. Each of the endothelium-intact and denuded aortic ring preparations was suspended between the bases of 2 triangular-shaped wires. One wire was attached to a fixed tissue support in a 10 ml isolated tissue bath containing Krebs solution bubbled with 95% O₂/5% CO₂ and maintained at 37°C [2–4]. The other end of each wire was attached by a cotton thread to a Grass FT.03 force displacement transducer connected to a Grass Model 7 polygraph. Isometric tension of the suspended rings was recorded on the polygraph via the transducers. The tissues were equilibrated for 90 minutes under a basal tension of 2g [2–4]. During this period, the bathing solution was changed every 20 minutes.

Subsequent to equilibration, the viability of tissues was tested by contracting them with a single (60 mM) concentration of KCl. After washing and further equilibration for 60 minutes, cumulative concentration-contraction curves for NE and KCl were obtained individually in endothelium-denuded preparations. In another set of experiments, cumulative concentration-relaxation curves for Ach and SNP were determined in PE (10⁻⁷M)-contracted endothelium-intact and mechanically-denuded aortic rings, respectively. To further investigate the possible mechanisms of the endothelium-mediated vasorelaxation, the responses to Ach were determined after pre-treating the intact vessels with the NO synthase inhibitor, N-nitro-L-arginine methyl ester (L-NAME, 10⁻⁴M), for 20 minutes [1, 3]. Tissue preparations were washed with Krebs solution and equilibrated for at least 60 minutes between each concentration-response curve for both the contractile and relaxant agents. This protocol did not affect the reactivities of the aortas to the tested vasoactive agents. After completion of each experiment, tissues were blotted and weighed.

Contractile responses of aortas to NE and KCl were determined as an increase in tension (g) in response to each drug concentration per mg tissue weight. Relaxant responses to Ach and SNP were calculated as the percentage of reduction in tension produced by PE in response to each concentration of the relaxant drugs.

Langendorff heart preparation and ischemia-reperfusion injury-related studies

The isolated hearts were perfused in the Langendorff mode using an isolated heart perfusion apparatus. The perfusion medium was standard Krebs-Henseleit buffer (37°C) containing 11 mM glucose and equilibrated with 95% O₂-5% CO₂ [21]. The regional model (i.e., coronary ligation) of ischemia-reperfusion injury using the Langendorff perfused heart has been previously described [21–22]. Accordingly, during a 25-minute stabilizing period, a 2-0 silk suture was loosely placed around the left main coronary artery and passed through a small vinyl tubing to form a snare. After the stabilization period, coronary occlusion was affected by pulling the suture through the snare with the use of a customized vascular clamp; each heart was subjected to 40 minutes of regional ischemia followed by 1 hour of reperfusion. The experiment was terminated by retightening the snare and infusing a 0.5% solution of 1 to 10 µm zinc-cadmium fluorescent particles into the aorta (Duke Scientific). The fluorescent particles delineated the risk zone, which was observed with a 366-nm fluorescent lamp. Thereafter, the atria and great vessels were removed and the ventricles weighed and frozen before being cut into 2-mm-thick slices using a heart matrix and a sharp blade. The slices were then incubated for a period of 15 minutes at room temperature in a solution of 1% triphenyltetrazolium chloride in phosphate buffer (pH: 7.4). The stained slices were stabilized on a glass plate and covered with clear cellulose paper. Infarct size was expressed as the ratio of infarcted zones (lacking staining with tetrazolium) to risk zones (excluding

zinc cadmium particles), which were determined by computerized planimetry. At specific intervals during the ischemia-reperfusion protocol, the coronary flow rate was measured. Cardiac function was measured with a pressure transducer by inserting a 23 gauge needle through the ventricular wall; the pressure transducer was connected to a computerized heart performance analyzer (MicroMed, Louisville, KY).

Drugs and chemicals

Phenylephrine (PE), NE, Ach, SNP and L-NAME were all purchased from Sigma Aldrich (St. Louis, MO, USA). Stock solutions of all drugs were prepared by dissolving in distilled water at a concentration of 10^{-2} M [2–4]. Ascorbic acid at a concentration of 1 mg/ml was included in the solution containing NE in order to reduce the oxidation of NE. The stock solutions of drugs were diluted to appropriate concentrations with Krebs solution whenever needed.

Statistical analysis

Results are expressed as means $\pm$ SEM. Statistical differences between Cr(pic)3-supplemented and control groups were evaluated using Student t-test and considered to be significant if $p < 0.05$ [2]. StatMost 32 software (DataMost Corporation, South sandy, UT) was used for statistical analyses.

Results

Body weights of the SHR supplemented with Cr(pic)3 were similar to those of the controls at the time of sacrifice (body weight of SHR supplemented with Cr(pic)3 vs. control: 280 ± 4 vs. 272 ± 7 g; $n=6$ per group).

Effect of Cr(pic)3-treatment on blood pressure

Dietary Cr(pic)3 did not alter blood pressure of the SHR after either 3 or 6 weeks of supplementation relative to that of untreated animals; however, after 6 weeks of treatment the Cr(pic)3-treated SHR showed a tendency for higher ($p=0.0725$) diastolic blood pressure (Figure 1A, 1B).

Effect of Cr(pic)3-treatment on aortic contractile responses to NE and KCl

In aortas from both Cr(pic)3-treated and control SHR mechanically denuded of the endothelium, both NE and KCl produced concentration-dependent contraction (Figure 2A, 2B). However, no significant differences were observed between the two groups of tissues in their responsiveness to either NE or KCl.

Effect of Cr(pic)3-treatment on aortic relaxant responses to SNP and Ach

In both Cr(pic)3-treated and control SHR, SNP evoked similar concentration-dependent relaxation of PE-contracted aortic preparations that were denuded of endothelium (Figure 3A). Likewise, in endothelium-intact aortas from both Cr(pic)3-supplemented and control SHR, Ach induced relaxation of PE-contracted tissues in a concentration-dependent fashion (Figure 3B). However, the relaxant responses of the intact tissues to Ach were significantly ($p < 0.05$) enhanced in the Cr(pic)3-treated animals compared to the untreated controls. Incubation of the aortas with L-NAME caused a marked rightward shift in the concentration-response curves for Ach in both Cr(pic)3-treated and control groups (Figure 3B). Nonetheless, L-NAME produced greater rightward shift of the Ach concentration-response curve in rats treated with Cr(pic)3, eliminating the difference observed between the control and treated animals.

Effect of Cr(pic)3-treatment on myocardial susceptibility to ischemia-reperfusion injury

Ventricular (right and left) weight to body weight ratio was greater in the Cr(pic)3-treated than untreated SHR (5.6 ± 0.2 vs. 4.8 ± 0.1 mg/g, $p < 0.05$). Baseline coronary flow rate (Figure 4A, Time 0) and cardiac work, indexed by the rate-pressure-product (RPP; Figure 4B), were similar between the two groups. Baseline myocardial contractility (indexed as maximum $+dP/dt$) and myocardial relaxation (indexed as maximum $-dP/dt$) were greater for the Cr(pic)3-treated than untreated SHR (2277 ± 226 vs. 1521 ± 223 mmHg/sec, $p < 0.05$ and 1652 ± 180 vs. 1148 ± 157 mmHg/sec, $p = 0.062$, respectively). In both groups, induction of regional ischemia was associated with significant ($p < 0.05$) and similar reductions in coronary flow rate (Figure 4A), and similar percent reductions compared to baseline values in myocardial contractility and relaxations (Figure 5A, 5B). Restoration of coronary circulation was associated with significant increases in coronary flow rates lasting about 15 minutes followed by decline towards values achieved by the end of the ischemic phase; however, the increase in coronary low rate during reperfusion was greater for the Cr(pic)3-treated SHR (Figure 4A). While reperfusion was associated with further deterioration of myocardial contractility and relaxation in the untreated SHR, the Cr(pic)3-treated SHR displayed better recovery of both parameters, thereby resulting in a significant differential between the two groups (Figure 5A, 5B). Nonetheless, infarct size was similar between the untreated and Cr(pic)3-treated SHR (Figure 5C).

Discussion

We examined the effects of chronic Cr(pic)3 treatment of SHR on blood pressure, reactivity of aortas and the susceptibility of the myocardium to acute IRI. With respect to vascular effects, the results indicate that treatment with Cr(pic)3 does not affect a) the course or extent of hypertension in SHR, b) the contraction of endothelium-denuded aortas in response to either NE or KCl and c) SNP-induced relaxant response of endothelium-denuded aortas. However, the treatment causes enhancement of Ach-mediated relaxation of endothelium-intact aortas, an effect which was blocked by inhibition of NO synthase. Assessment of cardiac effects indicates that Cr(pic)3-treated SHR show increased recovery of coronary flow and myocardial performance despite similar infarct size following IRI compared to their untreated counterparts. Collectively, the results suggest that Cr(pic)3 treatment of SHR enhances NO-mediated relaxant responses of aortas and improves coronary circulation and functional recovery of the myocardium following IRI.

Vascular effects of Cr(pic)3

Chronic treatment of SHR with Cr(pic)3 did not affect the contraction of endothelium-denuded aortas in response to either NE or KCl. This indicates that the contractile ability of the aortic smooth muscle, whether initiated by receptor activation or depolarization, is not altered under these conditions. In addition, the treatment did not affect SNP-induced relaxation of endothelium-denuded vessels, suggestive of lack of an effect on the aortic smooth muscle. The documented lack of effect of the supplement on aortic smooth muscle responsiveness is consistent with its ineffectiveness to markedly affect blood pressure of the SHR. In support of this observation, Kopilas et al. [17] have also reported insignificant effect of Cr(pic)3 on blood pressure of these animals. On the other hand, the lack of effect of Cr(pic)3 on NE and KCl-induced contraction are in contrast to those of Proctor et al [27] who reported reduced PE-induced contractile responses of endothelium-intact aortas from Cr(pic)3-supplemented ($80 \mu\text{g/kg/day}$ chromium for 9 weeks starting at 4 weeks of age), insulin-resistant JCR:LA-cp rats. The reason(s) for the seemingly divergent observations may relate to differences in animal models (SHR vs. JCR:LA-cp rats), tissue preparations (endothelium-denuded aortas vs. endothelium-intact aortas) and/or dose and duration of Cr(pic)3 treatment.

To determine the potential impact of Cr(pic)3 on the endothelium, we measured Ach-induced relaxation of endothelium-intact aortic rings. The results demonstrate that these tissues responded with greater Ach-induced relaxation than preparations from untreated SHR. This observation supports our hypothesis that Cr(pic)3 enhances endothelial function and thus leads to augmentation of endothelium-mediated vasodilatory responses. The fact that the enhanced endothelium-dependent vasorelaxation of the tissues from Cr(pic)3-treated rats was abrogated to control level by L-NAME further suggests that the response involved the release of NO from the endothelium [1, 3]. It is unlikely that the difference between the two groups of SHR with regard to aortic endothelial responses is related to the magnitudes of preceding contraction of the tissues because there was no difference in their contractile responsiveness to PE (data not shown). Since the responses of the aortic smooth muscle to SNP were unaltered by Cr(pic)3, variation in tissue NO-sensitivity was also unlikely to be a contributory factor. Our observation that the concentration of L-NAME used was able to inhibit only a portion of the Ach-induced relaxation suggests that, besides NO, other mediators released from the endothelium are involved in the response of the tissue, and based on the literature, these are likely to include at least prostacyclin and endothelium-derived hyperpolarizing factor [1, 28].

The greater endothelium-dependent tissue relaxation caused by oral Cr(pic)3 supplementation in SHR is consistent with previous observations of increased relaxation of endothelium-intact aortas from Cr(pic)3-treated JCR:LA-cp rats in response to Ach [8, 27] and enhanced vasodilatory responses of mesenteric arteries from sucrose-fed, Cr(pic)3-treated SHR to Ach [17]. Also, similar to our findings, the endothelial effect of Cr(pic)3 was found to be linked to augmented NO signaling, and the relaxant responses of the aortic smooth muscle to SNP were unaffected by the supplement [27]. As noted earlier, the lack of effect of Cr(pic)3 on the vascular smooth muscle responses to SNP provides additional support that the enhanced Ach-induced vasorelaxation observed in the intact rings from Cr(pic)3-treated rats is mediated solely via the endothelium. It is, however, unclear why Cr(pic)3 produced augmented endothelial responses in either the SHR or JCR:LA-cp rats [27]. However, based on previous indirect evidence, it may be related to the availability of calcium for NO release in endothelial cells and/or the antioxidant property of the supplement (9, 19, 25).

The lack of an effect of Cr(pic)3 on blood pressure of the SHR and the augmenting effect of the supplement on endothelium-mediated aortic responses suggests that the response of the endothelium does not contribute sufficiently enough to lower blood pressure. However, given the overall beneficial effects of the endothelium/nitric oxide in blood vessel functions, it can be postulated that Cr(pic)3, via its action on the endothelium, may have a subtle vasoprotective role as the animals develop increased blood pressure [3, 9].

Cardiac effects of Cr(pic)3 treatment

A noted finding of this study with regard to the myocardium is that, despite similar infarct size, hearts from Cr(pic)3-treated SHR showed improved recovery of coronary circulation and myocardial function following ischemia reperfusion insult, compared to untreated SHR. The apparent uncoupling between cell death resulting from the effects of ischemia-reperfusion and functional recovery of the myocardium has been seen under a variety of conditions and is likely to be due to different cellular pathways that mediate the demise of the cell and those involved in functional recovery of viable portions of the myocardium [21]. The observed functional effects may relate to a number of factors. First, better restoration of coronary circulation and the ensuing “wash out effect” could facilitate greater amelioration of lactic acidosis secondary to the ischemic episode. Second, Cr(pic)3 could beneficially influence dysregulation of calcium homeostasis in the ischemic reperfused myocardium. This notion is supported by a) the demonstration that the myocardium accumulates trivalent

chromium following Cr(pic)3 treatment [18] and b) Cr(pic)3 treatment reverses depressed sarcoplasmic reticulum Ca^{2+} ATPase activity and $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger expression in cardiac myocytes from ob/ob mice [14]. Third, Cr(pic)3 purportedly possesses antioxidant property that could be beneficial to the myocardium subjected to IRI [14, 25].

It is noteworthy that a recent study examined the effect of an energy formula containing chromium on the outcome of myocardial IRI in normotensive Sprague-Dawley rat [29]. This energy drink, along with other ingredients, contained chromium in the form of niacin-bound-chromium, a formulation that reportedly increases the bioavailability of trivalent chromium. The results indicate that hearts of animals consuming the high energy formula exhibited increased resistance to IRI as demonstrated by superior postischemic functional recovery and reduced infarct size. The cardioprotective effects of the energy formula were associated with greater preservation of cellular energy stores; the authors attributed the beneficial effects of the energy formula primarily to the niacin-bound-chromium component [29]. This contention is further supported by a more recent study from the same group who showed that treatment of streptozotocin-induced diabetic rats with niacin-bound-chromium also improves recovery of left ventricular function and decreases infarct size [24]. Although we did not observe an infarct sparing effect of Cr(pic)3 treatment in SHR, we did observe improvement in functional recovery following IRI. The disparity in relation to infarct size may relate to differences in animal models, type of chromium formulations, dose and duration of study and technical differences, among others. Nonetheless, collective observations point to the need to further explore potential beneficial effects of trivalent chromium on comorbidities associated with type 2 diabetes and obesity, conditions for which the use of trivalent chromium compounds have often been advocated.

Conclusions

Oral Cr(pic)3 supplementation of SHR does not cause significant alterations in blood pressure or aortic smooth muscle contraction and relaxation. Similarly, baseline coronary flow and myocardial performance are not adversely affected by the treatment. Rather, Cr(pic)3 treatment is associated with improved recovery of coronary circulation and myocardial performance following acute ischemia reperfusion insult. Since Cr(pic)3 also augmented NO-mediated endothelium-dependent vasorelaxation, further investigation of the potential protective effect of this nutritional supplement on the cardiovascular system is warranted.

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List of abbreviations

Cr(pic)3	chromium picolinate
SHR	spontaneously hypertensive rats
NE	norepinephrine
PE	phenylephrine
Ach	acetylcholine
SNP	sodium nitroprusside
NO	nitric oxide

L-NAME N-nitro-L-arginine methyl ester
IRI ischemia reperfusion injury

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Figure 1. Effect of dietary chromium picolinate [(Cr(pic)3)] supplementation on mean arterial pressure (MAP), systolic blood pressure (SAP) and diastolic blood pressure (DBP) of spontaneously hypertensive rats (SHR). Animals were treated with (Cr(pic)3-enriched diet containing 10 mg/kg chromium for a period of 3 weeks (panel A) and 6 weeks (panel B) starting at 6 weeks of age; control animals were given standard rat diet. Each bar represents mean $\pm$ SEM values from 6 rats/group.

Figure 2. Contractile responses of endothelium-denuded aortas from animal groups described in Figure 1 to norepinephrine (NE) (panel A) and potassium chloride (KCl) (panel B). Each point represents the mean $\pm$ SEM of data from 9–15 aortic rings obtained from 6 rats/group.

Figure 3.

Panel A shows relaxant responses of endothelium-denuded aortas from animal groups described in Figure 1 to sodium nitroprusside (SNP). Each point represents the mean $\pm$ SEM of data from 6 rats/group. Panel B shows relaxant responses of endothelium-intact aortas from animal groups described in Figure 1 to acetylcholine (ACh) in the absence and presence of N-nitro-L-arginine methyl ester (L-NAME). Each point represents the mean $\pm$ SEM of data from 6–16 aortic rings obtained from 6 rats/group. * $p < 0.05$ with respect to corresponding control values in the absence of L-NAME.

Figure 4. Line graphs showing coronary flow rate (normalized to ventricular weight (vW); panel A) and bar graphs show rate-pressure-product (RPP, an index of cardiac work; panel B) in animal groups as described in Figure 1. In panel A, Time 0 values represent baseline coronary flow rate. Each value represents the mean $\pm$ SEM of data from 6 rats/group. * $p < 0.05$ compared to the untreated SHR at the same time point.

Figure 5.

Bar graphs showing indices of myocardial contractility (maximum + dP/dt; panel A) and relaxation (maximum - dP/dt; panel B) expressed as percent of baseline values at the end of ischemia and reperfusion for animal groups as described in Figure 1. Also shown is infarct size, expressed as percent of area at risk, for Cr(pic)3-treated and control SHR (panel C). Each value represents the mean $\pm$ SEM of data from 6 rats/group. * $p < 0.05$ compared to the untreated SHR. # $p < 0.05$ compared to their ischemic values.