Increased Infarct Size in Uremic Rats: Reduced Ischemia Tolerance?

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Abstract. In patients with renal failure, myocardial infarction (MI) is more frequent and the rate of death from acute MI is very high. It has been argued that ischemia tolerance of the heart is reduced in uremia, but direct evidence for this hypothesis has not been provided. It was the purpose of this study (*1*) to ligate the left coronary artery and to measure the nonperfused area (risk area: total infarction plus penumbra) as well as the area of total infarction in subtotally nephrectomized (SNX) rats compared with sham-operated pair-fed control rats and (*2*) to examine the effects of potential confounders such as BP, sympathetic overactivity, and salt retention. The left coronary artery was ligated for 60 min, followed by reperfusion for 90 min. For visualizing perfused myocardium, lissamine green ink was injected. The nonperfused area (lissamine exclusion) and the area of total infarction (triphenyltetrazolium chloride stain) were assessed in sections of the left ventricle using image analysis. Groups of SNX rats also received: antihypertensive treatment (nadolol plus hydralazine); moxonidine; high salt diet or low salt diet (1.58% *versus* 0.015%). In surviving animals, the nonperfused area at risk (as the proportion of total left ventricular area), presumably determined by the geometry of vascular supply, was similar in sham-operated and SNX animals (0.38 ± 0.13 *versus* 0.45 ± 0.09; NS). In contrast, the infarcted area, given as a proportion of the nonperfused risk area, was significantly ($P < 0.003$) higher in SNX (0.68 ± 0.09) compared with sham-operated (0.51 ± 0.11) rats and was not altered by any of the above interventions. The finding that a greater proportion of nonperfused myocardium undergoes total necrosis is consistent with the hypothesis of reduced ischemia tolerance of the heart in renal failure. The findings could explain the high rate of death from MI in patients with impaired renal function.

The high cardiovascular mortality of renal patients has been widely appreciated since the seminal communication of Lindner (1). This is undoubtedly, at least in part, the consequence of premature and more severe atherosclerosis of the coronary arteries (2,3). Such accelerated atherogenesis recently also was documented in experimental models (4).

Not only is the frequency of myocardial infarction (MI) increased in renal patients, there is also convincing evidence that the rate of death from acute MI is dramatically increased. In the observation of Shlipak *et al.* (5), even moderate renal insufficiency was associated with a substantially elevated risk of death during the first months of follow-up after MI. In a retrospective cohort study on patients with acute MI, Wright *et al.* (6) found a graded increase of in-hospital mortality with decreasing renal function. Mortality was 2% in patients with normal renal function and 30% in patients with ESRD. Although the authors commented on the "association between reduced use of acute perfusion therapy in these patients and poor survival" (6), there is little doubt that the rate of death from MI is intrinsically increased in renal disease. Surprising is that no information on the size of the infarcts is currently available.

To obtain experimental information on the size of MI, we selected a model of coronary ligation that had been shown to yield standardized outcomes with respect to the area of total infarction (7,8). We explored whether in subtotally nephrectomized rats the hypothetical reduction in ischemia tolerance (9,10) would cause a greater area of total necrosis in the left ventricle (LV). In this model, the nonperfused volume corresponds to the vascular territory supplied by the descending branch of the left coronary artery. Although the vascular territory is unlikely to change in a short-term study, we also assessed the nonperfused risk area, *i.e.*, the composite of the area of total infarction plus the area of ischemic tissue damage. The latter is the so-called penumbra. To exclude artifacts from potential confounders such as hypertension, sympathetic overactivity, and salt retention, groups of subtotally nephrectomized rats with interventions—BP lowering, moxonidine administration, and high salt *versus* low salt diet—were investigated as well.

Materials and Methods

Animals

Twelve-week-old male Sprague Dawley rats (150 to 200 g; Ivanovas Co., Kisslegg, Germany) were maintained in single cages under

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conditions of constant temperature and humidity. The animals had free access to ssniff R/M-H V1535 pellets (19% protein, 0.25% sodium; http://www.ssniff.de). After 1 wk of adaptation, the animals were subjected to either two-step surgical subtotal nephrectomy (SNX) or sham corresponding operation (11). In brief, animals were anesthestized with 0.02 ml of xylazine (Rompun 2%; Bayer Co., Leverkusen, Germany) and 0.2 ml of ketamine (Ketanest 10%; WDT, Garbsen, Germany). In a first operation, the right kidney was decapsulated (sham operation) with or without subsequent nephrectomy. The removed kidney was weighed. After 1 wk, the cortex of the left kidney was subtotally resected. The resected tissue was weighed, and an amount of cortex corresponding to two thirds of the weight of the right kidney was removed. Sham-operated and SNX animals, matched for body weight, were subjected to a pair-feeding protocol, in which the matched sham-operated animal received exactly the amount of food consumed on the preceding day by the matched SNX partner. The duration of the experiment was 3 wk. BP was measured by tail plethysmography at weekly intervals.

In an initial study (series 1), pair-fed SNX animals were compared with sham-operated animals. In a follow-up study (series 2), three interventions were investigated. In a first comparison (series 2/experiment 1), untreated SNX rats were compared with SNX rats that received antihypertensive treatment (hydralazine and nadolol in the drinking water), concentrations being adjusted to deliver a daily dose of 50 and 10 mg/kg per d, respectively; the two groups were on a pair-feeding protocol. In addition, in a second comparison, a group of SNX rats, similarly pair fed with the SNX untreated rats of experiment 1, received moxonidine (Lilly Company, Hannover, Germany) in the drinking water, delivering a dose of 1.5 mg/kg per d (series 2/experiment 2) and compared with the untreated SNX rats of experiment 1. Finally, in a third arm, SNX rats were maintained after the second operation on either a low-salt (0.015%) or a high-salt (1.58%) diet (18% protein; Altromin C1036 and C1051, respectively) using a pair-feeding protocol (series 2/experiment 3).

Measurements

Under anesthesia with barbiturate (Ketanest 0.1 mg/kg), a catheter was implanted into the carotid artery (for online BP measurement) and jugular vein (to administer saline 3 ml/h). The animals received tracheal intubation and artificial ventilation. The thoracic cage was opened by sternal resection, the pericardium was incised, and the left coronary artery was ligated for 60 min. Subsequently, the ligature was opened and the heart was reperfused at controlled BP for 90 min. Subsequently, the ligature was again applied and a bolus of lissamine-green ink (1 ml) was injected into the jugular vein to visualize the perfused myocardium. After 30 s, the heart was removed and prepared as described previously (8). In brief, the heart was sectioned at 2-mm intervals. Photographs of the two opposite surfaces of the section were obtained to quantify the nonperfused area that, by definition, excluded lissamine-green ink. For identifying the area of total infarction, slices were incubated with triphenyltetrazolium chloride (TTC; 0.5 mg/ml phosphate buffer for 20 min at 37°C). In the presence of intact dehydrogenase enzyme systems, TTC forms a brownish precipitate, whereas areas of necrosis lack dehydrogenase activity and do not stain. Consequently, areas not stained with TTC correspond to areas of total necrosis. Figure 1 shows a representative heart preparation to illustrate the technique.

The respective areas (total LV area, nonperfused area at risk, area of total necrosis) were quantified using a computer-based program. The sections were put on a coverslip and photographed with a digital camera. The respective areas were then analyzed using a computer-based system (Adobe Photoshop, Adobe Inc. and NIH Image, a public domain image processing and analysis program; http://rsb.info.nih-.gov). The investigator involved was blinded to the group assignment of the animals. The error of replicate measurements was 4.31 ± 0.62% in 24 measurements of five animals each.

Ancillary Measurements

Hemoglobin (Hb), urea, and creatinine were measured with auto-analyzers (Celldyne Abbot Co. and LX 20 Beckman Co., respectively).

Statistical Analyses

Data are given as mean ± SD. After a check for Gaussian distribution, the data were analyzed using the *t* test for pair differences

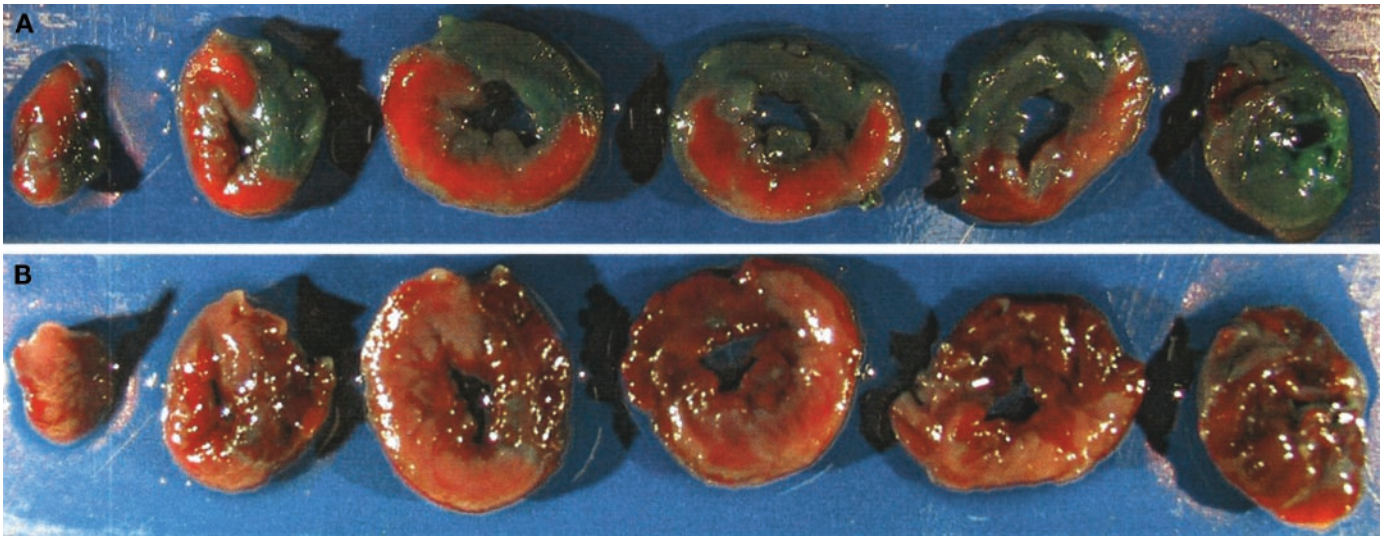

Figure 1. For demonstrating the technique, representatives sections perpendicular to the long axis of the left ventricle (LV) are shown. (A) The green area indicates that the tissue had been perfused by Lissamine green ink *in vivo*. The red segment shows LV tissue that had been excluded from perfusion constituting a nonperfused area at risk. (B) Tetrazolium stain is an indicator of mitochondrial respiration; the brownish stain indicates non-necrotic tissue with preserved mitochondrial oxidation. With this technique, pale areas are areas of total necrosis.

(using SPSS statistical analysis program version 11.5; Munich, Germany). The data for the primary end point (the ratio infarcted area/nonperfused area at risk) were considered as significant when the working hypothesis was rejected at $P < 0.05$. For excluding potential artifacts from multiple comparisons, the significance was also tested after Bonferroni correction.

Results

Animal Data

Despite similar initial body weight, SNX rats in series 1 had lower final body weights, although the pair-feeding protocol guaranteed identical food intake. Systolic BP values as well as serum urea and creatinine concentrations were significantly higher in SNX rats, and the Hb concentrations were significantly lower (Table 1). In series 2, untreated SNX rats were compared with SNX rats that were subjected to different interventions as described in Material and Methods and specified in Table 2.

The final body weight was comparable in all groups. Systolic BP was significantly lower in animals with antihypertensive medication (experiment 1), significantly lower in animals on moxonidine (experiment 2), and significantly higher in SNX rats on a high-salt diet (experiment 3). There were no significant differences in S-urea concentration.

Cardiac Findings in Series 1: Comparison of Sham-Operated Pair-Fed and SNX Rats

Nonperfused area at risk. The ratio LV weight/body weight was significantly higher in SNX animals, documenting LV hypertrophy. The nonperfused area at risk, *i.e.*, the lissamine-green negative area, given as the proportion of the total area of the LV was similar in sham-operated pair-fed controls and SNX rats, respectively. In contrast, the area of total necrosis, *i.e.*, the area staining negative for tetrazolium, indicating loss of mitochondrial oxidation, when given as a proportion of the nonperfused area at risk was significantly higher in SNX rats. The variation coefficients in the two groups were not significantly different (Table 3).

Area of total infarction. Figure 2 gives the individual values for the area of total infarction as a proportion of the nonperfused area at risk in sham-operated pair-fed control and SNX rats, respectively. The difference was highly significant ($P < 0.003$), and this remained significant even after Bonferroni correction for multiple comparisons, *i.e.*, also infarcted area/LV area.

All animals developed short-lived episodes of ventricular fibrillation (VF) after ligation, which could be mostly reversed by tapping the heart. All animals that developed hypotension died within minutes. The proportion of animals with therapy-resistant VF terminating in hypotension and death was 10% in sham-operated and 38.5% in SNX animals (but only 20% in SNX rats on nadolol). The LV of animals with irreversible VF could not be used for the analysis because this precluded perfusion with lissamine-green ink.

Cardiac Findings in Series 2: Comparison of Pair-fed SNX Animals with and without Interventions

There was no significant difference in the ratio LV weight/body weight in any of the experiments with the exception of experiment 3, in which the ratio was higher on the high-salt diet. All three interventions had no effect on the ratios nonperfused risk area/LV area or infarcted area/nonperfused risk area, respectively (Table 4).

Discussion

The salient finding of the present study is an observation that further supports the concept of reduced ischemia tolerance in uremia (9,10): in SNX rats, after ligation of the descending branch of the left coronary artery, the area of total necrosis (devoid of mitochondrial oxidation) was higher than in sham-operated pair-fed controls when expressed as a percentage of the nonperfused area at risk. The same is true when the area of total necrosis was expressed as a percentage of the total LV area. The finding is not explained by potential confounding effects of hypertension, sympathetic overactivity, and salt retention, although the last was accompanied by even more pronounced LV hypertrophy.

The finding that the nonperfused area at risk comprised a similar proportion of the total LV area and by implication LV mass is not unanticipated, because the size of the nonperfused area at risk reflects the vascular territory supplied by the left coronary artery. It was *a priori* not likely that this would change during the short experimental period. In SNX rats, the vascular territory presumably grew in proportion to the growth of the LV. In contrast, a greater proportion of the nonperfused area at risk underwent total necrosis. The nonperfused area was quantified by injecting lissamine-green, an agent that remains in the intravascular space; nonperfused tissue excludes lissamine-green. Subsequently, *ex vivo*, the tissue was stained with tetrazolium, an indicator of preserved mitochondrial oxidation; tetrazolium-negative tissue indicated total necrosis. Such han-

Table 1. Animal data (series 1): Sham-operated pair-fed controls *versus* SNX rats[a]

	Body Weight (g)	Systolic BP (mmHg)	S-Urea (mg/dl)	S-Crea (mg/dl)	Hb (g/dl)
Sham-operated pair-fed controls ($n = 9$)	370 ± 8.7	102 ± 10.9	54 ± 7	0.49 ± 0.16	14.2 ± 0.36
SNX pair-fed rats ($n = 9$)	318 ± 9.2	124 ± 10.8	101 ± 12.3	0.91 ± 0.20	11.8 ± 0.55
P	0.01	0.01	0.05	0.01	0.03

[a] SNX, subtotally nephrectomized; Hb, hemoglobin.

Table 2. Animal data (series 2): Untreated SNX *versus* SNX plus intervention

	Body Weight (g)	Systolic BP (mmHg)	S-Urea (mg/dl)	S-Crea (mg/dl)	Hb (g/dl)
Experiment 1					
SNX untreated pair-fed rats ($n = 8$)	322 ± 3.5	121 ± 18.1	101 ± 12.3	1.01 ± 0.18	11.8 ± 0.55
SNX + hydralazine/nadolol pair-fed rats ($n = 8$)	322 ± 3.5	77.5 ± 15.8	99.4 ± 7.1	1.08 ± 0.10	14.2 ± 0.88
P[a]	NS	0.01	NS	NS	0.04
Experiment 2					
SNX + moxonidine ($n = 7$)	317 ± 4.9	92.8 ± 7.5	90.2 ± 9.0	1.04 ± 0.16	14.4 ± 1.3
P[a]	NS	NS	NS	NS	0.02
Experiment 3					
SNX low-salt pair-fed rats ($n = 8$)	324 ± 7.4	99.4 ± 10.8	92.0 ± 8.2	1.20 ± 0.21	12.2 ± 1.9
SNX high-salt pair-fed ($n = 8$)	325 ± 71.6	128 ± 10.4	84.6 ± 10.6	1.00 ± 0.19	11.3 ± 0.9
P	NS	0.01	NS	NS	NS

[a] Pair-fed and compared with untreated SNX rats in experiment 1.

Table 3. Cardiac findings in sham-operated pair-fed controls and SNX rats (series 1)

	LV Weight/ Body Weight Ratio ($\times 10^3$)	Nonperfused Area at Risk/ LV Area	Infarcted Area/ LV Area	Infarcted Area/ Nonperfused Area at Risk
Sham-operated pair-fed controls ($n = 9$)	2.27 ± 0.24	0.38 ± 0.13	0.18 ± 0.06	0.51 ± 0.11
SNX pair-fed rats ($n = 9$)	2.87 ± 0.45	0.45 ± 0.09	0.3 ± 0.06	0.68 ± 0.09
P	0.04	NS	0.01	0.003

dling of the tissue with stains did not allow the study of pathomechanisms underlying the more marked necrosis by immunohistologic and molecular techniques.

The observation is on a solid biostatistical basis because the reproducibility of measurements was high and the coefficient of variation was relatively low in the two groups. Use of serial sections (see Figure 1), probing the entire LV, excluded artifacts that resulted from changes in LV geometry in SNX animals. A certain proportion of SNX animals developed irreversible VF, hypotension, and death. The exact reason for the high proportion of SNX rats' dying from VF is not known. It is interesting that in the group of SNX animals that were treated with a β-blocker, the proportion that developing VF was lower. This observation is consistent with but does not prove a role for sympathetic overactivity.

We emphasize that the model of subtotal nephrectomy that we selected does not lead to advanced uremia. In this model, the number of nephrons is reduced from ~30,000 to ~15,000 per animal with little interindividual variation (12). The low degree of renal dysfunction is not a disadvantage because the death rate after MI is increased in patients with even minor renal dysfunction (5,6). The low degree of renal dysfunction in our model presumably excludes a number of factors that might have an impact on the outcome in more advanced renal failure, such as metabolic acidosis, hyperkalemia, hyperparathyroidism, alterations in intracellular calcium homeostasis, etc.

In previous morphometric studies, we showed that in this model, the number of cardiomyocytes is decreased presumably as a result of apoptosis (13). Furthermore, the cardiomyocyte diameter and cardiomyocyte area are increased, whereas the growth of capillaries does not keep pace (11,14). Consequently, the length density of the capillaries is significantly lower in uremic compared with BP-matched control animals. The critical distance that oxygen has to diffuse from midcapillary into the center of the cardiomyocyte thus is increased and the cardiomyocyte is presumably at greater risk of hypoxia because diffusion is inversely related to the square of the distance. We do not have direct evidence for disturbed oxygen diffusion in this model, but this mechanism would be plausible. Therefore, we propose that the microcirculatory abnormalities of the heart (microvessel disease) diminish oxygen delivery and thus account primarily for the finding that the area of total necrosis is greater in uremic rats.

Nevertheless the possibilities of increased oxygen demand or insufficient metabolic compensation for hypoxia must also be considered. In the Langendorff heart preparation of uremic animals, hypoxia increased the cytosolic calcium concentration during diastole (10), possibly related to diminished sarcoplasmic Ca^{++}ATPase in uremia (15,16). Kennedy *et al.* (17) found altered calcium cycling and contractile function in isolated cardiomyocytes of uremic animals. The resulting higher wall stress will not fail to increase oxygen demand.

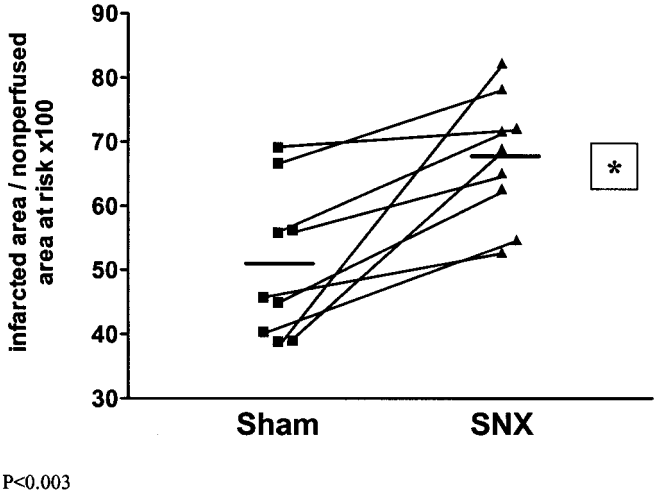

Figure 2. Ratio of area of total infarction/nonperfused area at risk. Matched sham-operated controls are compared with pair-fed subtotally nephrectomized rats. Values are of individual matched pairs and medians. *$P < 0.03$.

There are also indications that metabolic compensation for hypoxia is inadequate in uremia. Raine *et al.* (10) found instability of creatine phosphate and increased degradation of ATP to adenosine under low-flow conditions in hearts of uremic compared with control rats. In uremia, insulin-mediated glucose uptake by the heart is diminished (18). During hypoxia, generation of high-energy nucleotides by glycolysis requires more glucose, but glucose delivery is diminished as a result of insulin resistance. In diabetes with similar insulin resistance, death from MI (19,20) is increased and is dramatically lowered by administration of insulin plus glucose (20). Complement activation is a major factor aggravating destruction in areas of infarction (21,22). Increased concentrations of factor D in uremia (23) amplify the generation of the tissue-damaging membrane attack complex (24) and may also increase the size of necrosis.

We tried to exclude potential confounders accounting for the increased infarct size, such as hypertension, sympathetic overactivity, and salt retention. Hypertension is a potential confounder, because it is known that the risk of death from MI is greater in hypertensive compared with normotensive patients (25). In experimental models of hypertension, greater infarct sizes have also been observed. Therefore, we treated animals with hydralazine and nadolol in a dose that had previously been shown to lower BP and to block completely the isoproterenol-induced increase in heart rate (26). BP was lowered deliberately to levels below those in sham-operated animals, but even this did not significantly affect the infarct size. We confirmed the previous results of Rambausek *et al.* (26) that the LV weight/body weight ratio is also not significantly lowered by this intervention, suggesting that the increase in LV mass in SNX rats is BP independent, at least to a large extent.

We also assessed the effect of moxonidine on the basis of the consideration that renal injury causes a dramatic increase in sympathetic activity (27) and that low nonhypotensive doses of moxonidine decreased sympathetic activity and interfered with target organ damage in uremia (28). The dose of moxonidine administered lowered BP but failed to affect infarct size. We consider this a pilot experiment because the experimental protocol precluded measurements of blood or tissue catecholamines and assessment of catecholamine action.

In patients with essential hypertension, a high dietary intake of salt increases LV mass independent of BP (29,30), and

Table 4. Cardiac findings in untreated and treated SNX rats (series 2)

	LV Weight/ Body Weight Ratio ($\times 10^3$)	Nonperfused Area at Risk/ LV Area	Infarcted Area/ LV Area	Infarcted Area/ Nonperfused Area at Risk
Experiment 1				
SNX untreated pair-fed rats ($n = 8$)	2.46 ± 0.24	0.396 ± 0.093	0.295 ± 0.072	0.756 ± 0.093
SNX + hydralazine/ nadolol ($n = 8$)	2.35 ± 0.23	0.373 ± 0.073	0.27 ± 0.069	0.73 ± 0.094
P[a]	NS	NS	NS	NS
Experiment 2				
SNX + moxonidine ($n = 7$)	2.52 ± 0.31	0.40 ± 0.05	0.282 ± 0.04	0.69 ± 0.08
P[a]	NS	NS	NS	NS
Experiment 3				
SNX low-salt pair-fed rats ($n = 8$)	2.42 ± 0.27	0.355 ± 0.079	0.267 ± 0.048	0.772 ± 0.065
SNX high-salt pair-fed rats ($n = 8$)	3.15 ± 0.38	0.337 ± 0.079	0.264 ± 0.068	0.788 ± 0.081
P	0.01	NS	NS	NS

[a] Pair-fed and compared with untreated SNX rats in experiment 1.

sodium also increases LV mass in experimental animals (31). We reasoned that in the renal ablation model, a certain degree of salt retention would occur. Comparing SNX animals on low-salt and high-salt diet, we found greater LV mass but no change in relative infarct size. In other words, on high-salt diet, the SNX animals had greater LV weight/body weight ratio but the infarct size as a percentage of the area at risk was similar to that of SNX on low-salt diet. This observation argues against direct or indirect effects of salt loading on infarct size, *e.g.*, via hypervolemia and increased preload or via mechanisms such as local generation of angiotensin II, upregulation of AT-1–receptor density (32), upregulation of the endothelin system (33), salt-induced activation of the local aldosterone system (34), or oxidative stress (35). We acknowledge that in the intervention studies, we did not examine potential effects on nonuremic animals but consider potential artifacts as a result of this omission unlikely.

In conclusion, our results document that in animals with a modest degree of renal dysfunction, infarcts after coronary artery ligation are larger. This finding is unrelated to hypertension, sympathetic overactivity, or salt retention. To the extent that animal data can be extrapolated to humans, this finding may help to explain the high risk of death from MI in patients with early (5,6) and particularly with advanced (36–38) renal failure.

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