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Original article

Development of an experimentally useful model of acute myocardial infarction: 2/3 nephrectomized triple nitric oxide synthases-deficient mouse

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ABSTRACT

We investigated the effect of subtotal nephrectomy on the incidence of acute myocardial infarction (AMI) in mice deficient in all three nitric oxide synthases (NOSs). Two-thirds nephrectomy (NX) was performed on male triple NOSs$^{-/-}$ mice. The 2/3NX caused sudden cardiac death due to AMI in the triple NOSs$^{-/-}$ mice as early as 4 months after the surgery. The 2/3NX triple NOSs$^{-/-}$ mice exhibited electrocardiographic ST-segment elevation, reduced heart rate variability, echocardiographic regional wall motion abnormality, and accelerated coronary arteriosclerotic lesion formation. Cardiovascular risk factors (hypertension, hypercholesterolemia, and hyperglycemia), an increased number of circulating bone marrow-derived vascular smooth muscle cell (VSMC) progenitor cells (a pro-arteriosclerotic factor), and cardiac up-regulation of stromal cell-derived factor (SDF)-1α (a chemotactic factor of the progenitor cells) were noted in the 2/3NX triple NOSs$^{-/-}$ mice and were associated with significant increases in plasma angiotensin II levels (a marker of renin–angiotensin system activation) and urinary 8-isoprostane levels (a marker of oxidative stress). Importantly, combined treatment with a clinical dosage of an angiotensin II type 1 receptor blocker, irbesartan, and a calcium channel antagonist, amlodipine, markedly prevented coronary arteriosclerotic lesion formation and the incidence of AMI and improved the prognosis of those mice, along with ameliorating all those pro-arteriosclerotic parameters. The 2/3NX triple NOSs$^{-/-}$ mouse is a new experimentally useful model of AMI. Renin–angiotensin system activation, oxidative stress, cardiovascular risk factors, and SDF-1α-induced recruitment of bone marrow-derived VSMC progenitor cells appear to be involved in the pathogenesis of AMI in this model.

1. Introduction

Acute myocardial infarction is a disorder in which cardiac myocytes undergo necrosis as a consequence of interrupted coronary blood flow [1]. Acute myocardial infarction is a major cause of morbidity and mortality worldwide, with more than 7 million people in the world suffering from acute myocardial infarction each year [1]. Over the past two decades, the in-hospital mortality rate after admission for acute myocardial infarction has substantially declined to less than 10%, owing to

Abbreviations: ACE, angiotensin-converting enzyme; ADMA, asymmetric dimethylarginine; AMI, acute myocardial infarction; APC, activated protein C; apo E, apolipoprotein E; AT_1, angiotensin II type 1; CKD, chronic kidney disease; ECG, electrocardiography; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDL, high-density lipoprotein; mAb, monoclonal antibody; NO, nitric oxide; NOS, NO synthase; NX, nephrectomy; Sca-1$^+$, stem cell antigen-1$^+$; SDF-1α, stromal cell-derived factor-1α; VSMC, vascular smooth muscle cell; WHHL, Watanabe heritable hyperlipidemic; WT, wild-type.

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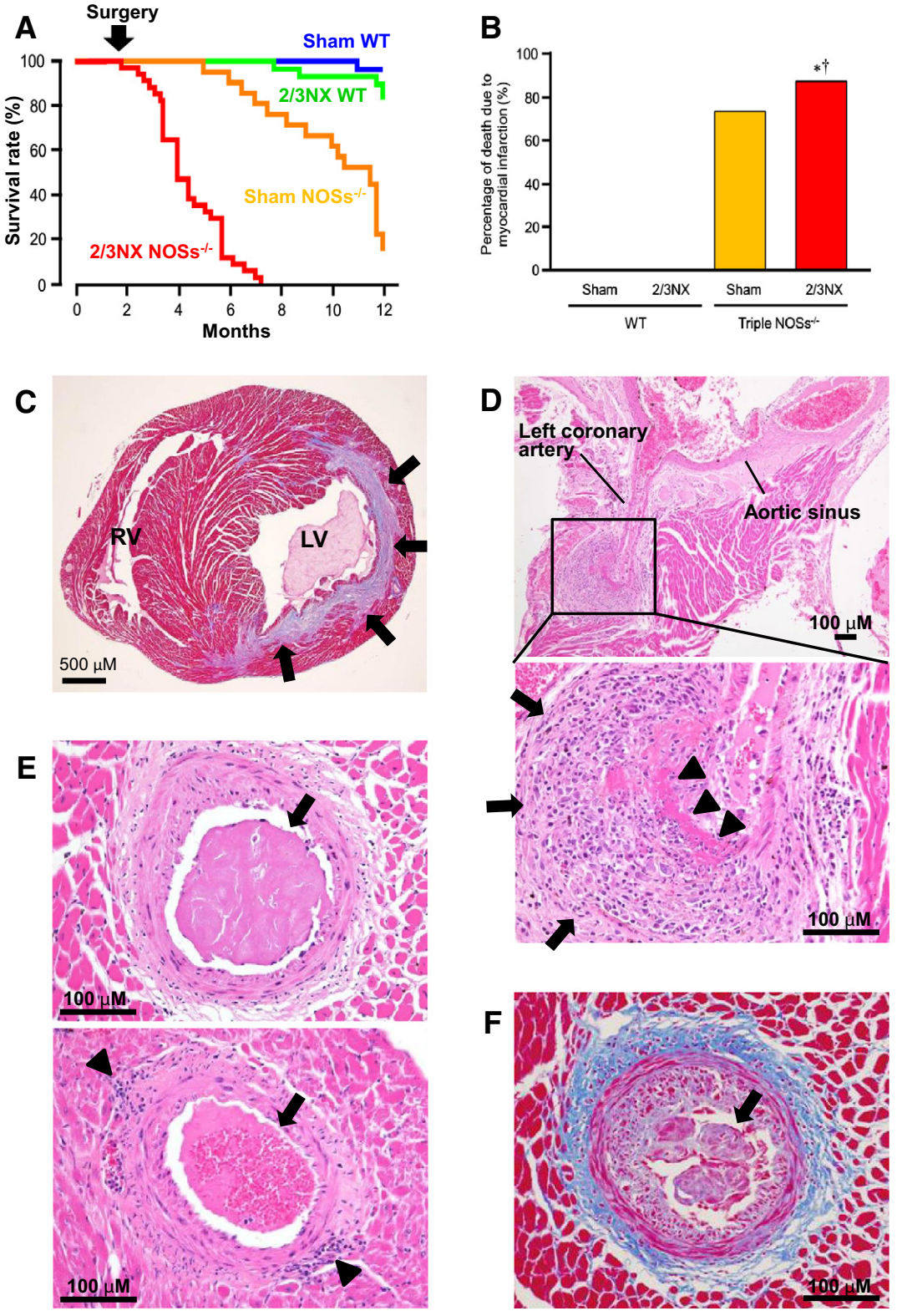


Fig. 1. Sudden cardiac death due to spontaneous myocardial infarction in 2/3 nephrectomized (NX) male triple nitric oxide synthases (NOSs)-deficient mice. (A) Survival rate ($n = 28$–49). NOSs$^{-/-}$, triple NOSs$^{-/-}$ mice; WT, wild-type mice; sham, sham-operated. (B) Percentage of death due to myocardial infarction in the total causes of death ($n = 2$–32). Sham, sham operation. (C) Lateral wall myocardial infarction (arrows) (Azan staining). LV, left ventricle; RV, right ventricle. (D) Marked infiltration of inflammatory cells (arrows) and fibrinoid necrosis (triangles) at the adventitia of the left coronary artery (hematoxylin-eosin staining). (E) Intracoronary thrombi (arrows) and adventitial infiltration of inflammatory cells (triangles) (hematoxylin–eosin staining). (F) Intimal thickening, perivascular fibrosis (blue color), and intracoronary thrombus (arrow) (Azan staining).

recent therapeutic advances such as coronary reperfusion therapy [2]. However, the overall mortality rate, including out-of-hospital deaths, is very high (approximately 30%) even at present [3]. This is because the majority of these deaths occur before stricken individuals reach the hospital [3]. Outside the hospital, once the individuals develop severe complications, such as malignant cardiac arrhythmia, cardiogenic shock, or cardiac rupture, it is extremely difficult to save their lives [3]. Thus, in order to suppress this fatal cardiovascular disorder, research and development of therapeutic strategies for preventing acute myocardial infarction are of critical importance. However, due to lack of an experimentally useful animal model that develops acute myocardial infarction, the research and development of such strategies have made little progress.

Nitric oxide (NO) plays an essential role in maintaining cardiovascular homeostasis. NO is synthesized by three distinct NO synthase (NOS) isoforms, including neuronal, inducible, and endothelial NOSs, and exerts a variety of biological actions under both physiological and pathological conditions [4–9]. We previously generated mice in which all three NOS genes are completely disrupted [10] and reported that triple NOSs$^{-/-}$ mice, but not single endothelial NOS$^{-/-}$ mice, spontaneously emerge acute myocardial infarction [11]. However, our model was not useful for experiments because it took a very long time (approximately 1 year) for them to develop acute myocardial infarction [11].

Chronic kidney disease (CKD) is a condition characterized by progressive and irreversible loss of renal function. It is estimated that over 10% of the adult population in developed countries suffer some degree of CKD [12,13]. Previous epidemiological studies have indicated that the presence of CKD significantly increases the risk of acute myocardial infarction in men, and that the impact of CKD on the risk of cardiovascular disease is as strong as that of diabetes mellitus and pre-existing ischemic heart disease [14–16]. In the clinical course of the progression of CKD, the number of nephrons decreases regardless of etiology, and this pathological renal remodeling is thought to be the final common

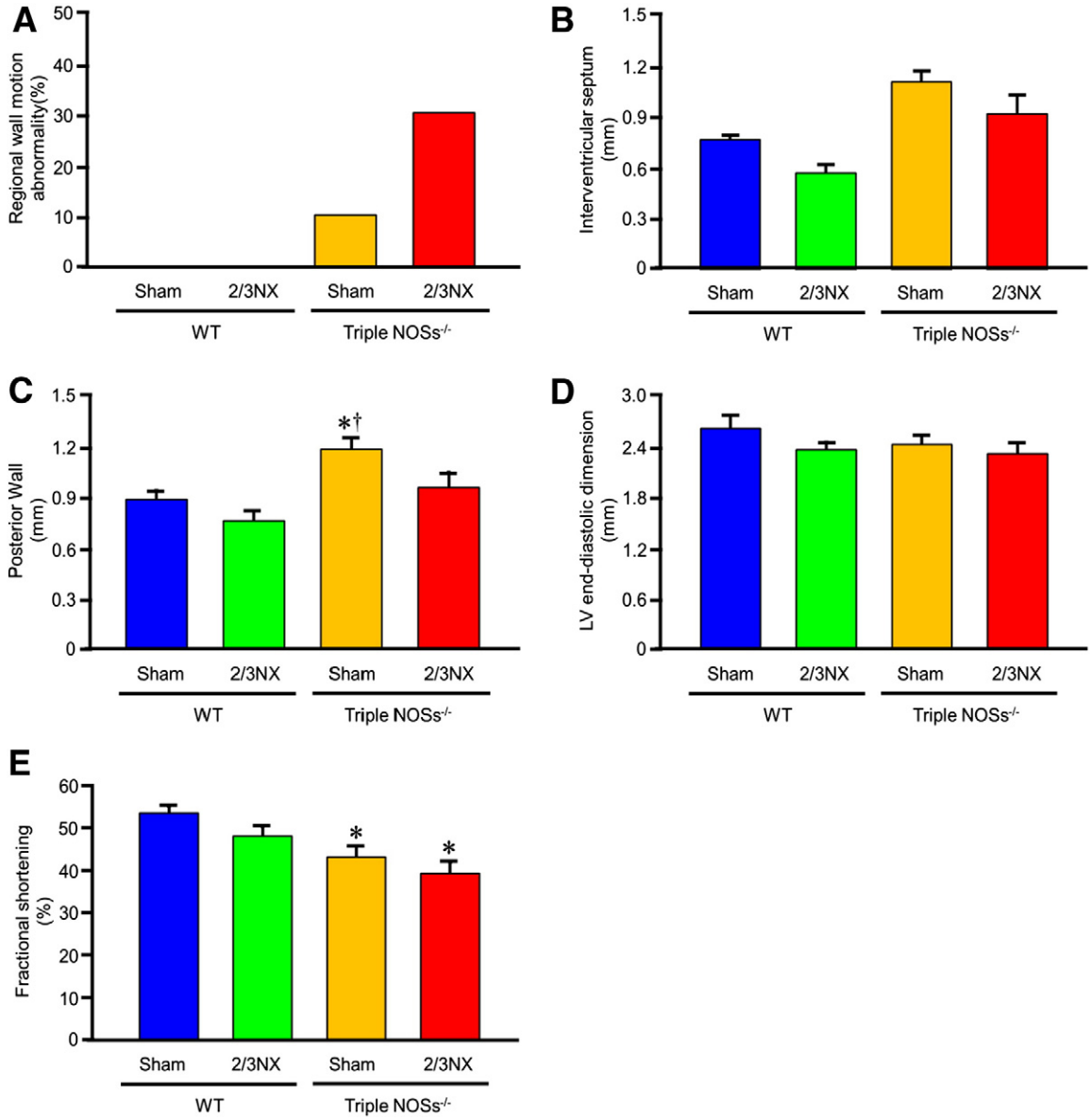


Fig. 2. Echocardiographic abnormalities in 2/3NX triple NOSs$^{-/-}$ mice at 2 months after the surgery. (A) Regional wall motion abnormality ($n = 10$). NOSs$^{-/-}$, triple NOSs$^{-/-}$ mice; WT, wild-type mice; sham, sham-operated. (B) Wall thickness of interventricular septum ($n = 10$). (C) Wall thickness of posterior wall ($n = 10$). (D) Left ventricular (LV) end-diastolic dimension ($n = 10$), (E) Fractional shortening ($n = 10$).

pathway in the pathogenesis of CKD. Such a disease state is modeled in experimental animals by surgically dissecting a large part of the renal mass [17,18].

In the present study, based on these backgrounds, we investigated the effect of subtotal nephrectomy on the incidence of acute myocardial infarction in our male triple NOSs$^{-/-}$ mice in order to establish an experimentally useful model of acute myocardial infarction.

2. Materials and methods

Materials and methods are described in the online Supplementary Methods and Results.

3. Results

3.1. Subtotal 2/3 nephrectomy (NX) caused an early onset of acute myocardial infarction in male triple NOSs$^{-/-}$ mice

Because animals with 5/6NX are widely used as an experimental model of CKD, we first studied the effect of 5/6NX on survival rate in male triple NOSs$^{-/-}$ mice. However, almost all the triple NOSs$^{-/-}$ mice died shortly after the 5/6NX (data not shown). Thus, we next examined the effect of 2/3NX. In male wild-type (WT) mice, the 2/3NX did not significantly affect the survival rate as compared with sham operation, and more than 80% of the 2/3NX WT mice lived during the 10 months of follow-up (Fig. 1A). In contrast,

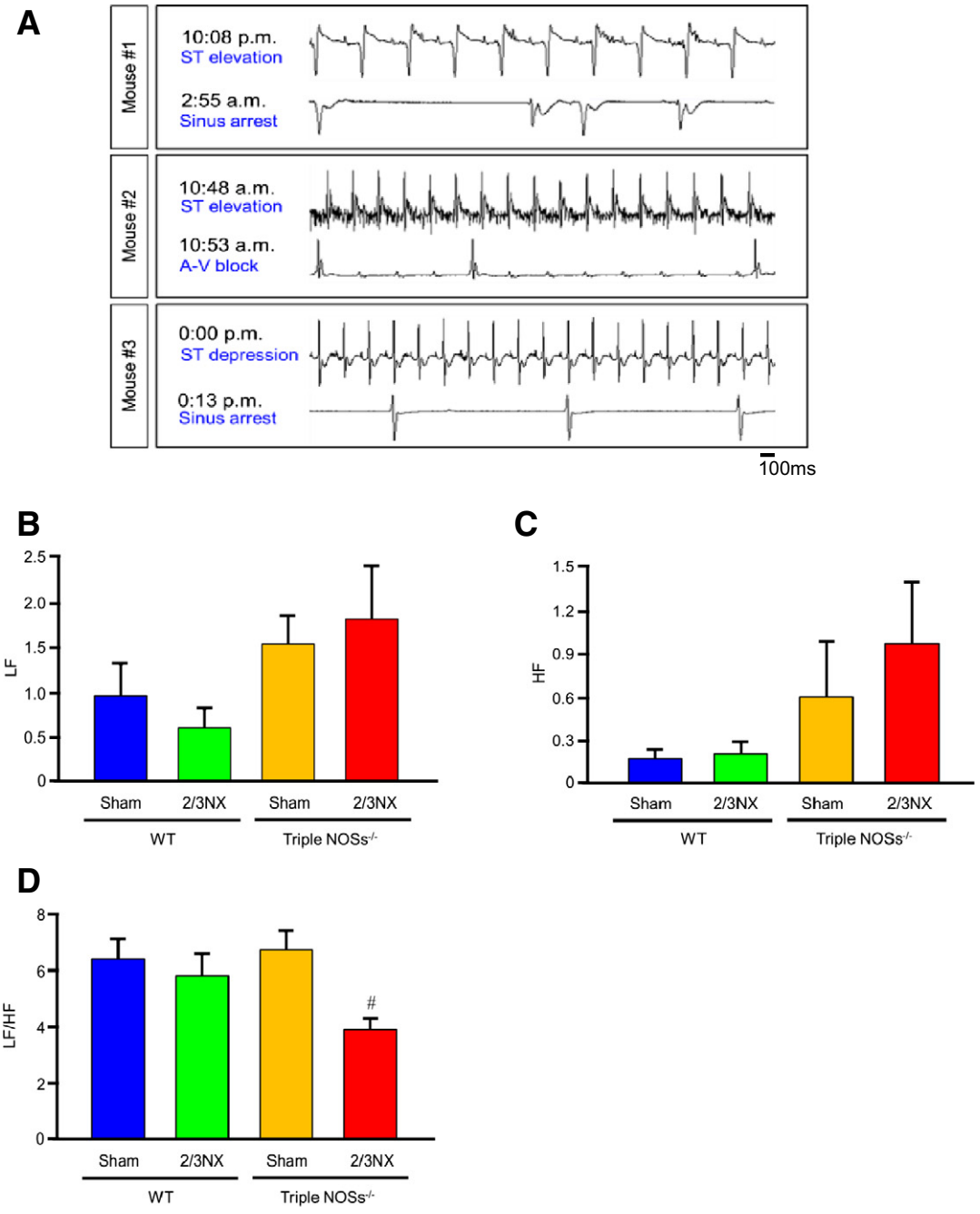


Fig. 3. Telemetry electrocardiographic abnormalities in 2/3NX triple NOSs$^{-/-}$ mice at 2 months after the surgery. (A) Electrocardiographic (ECG) abnormalities in 3 2/3NX triple NOSs$^{-/-}$ mice that died during ECG recording (died within 24 hours after subcutaneous implantation of telemetry transmitters). A-V, atrioventricular. (B) Low-frequency (LF) power ($n = 10$–12). (C) High-frequency (HF) power ($n = 10$–12). (D) LF/HF ratio ($n = 10$–12). $^{*}P < 0.05$ vs. sham-operated WT mice; $^{\#}P < 0.05$ vs. sham-operated triple NOSs$^{-/-}$ mice.

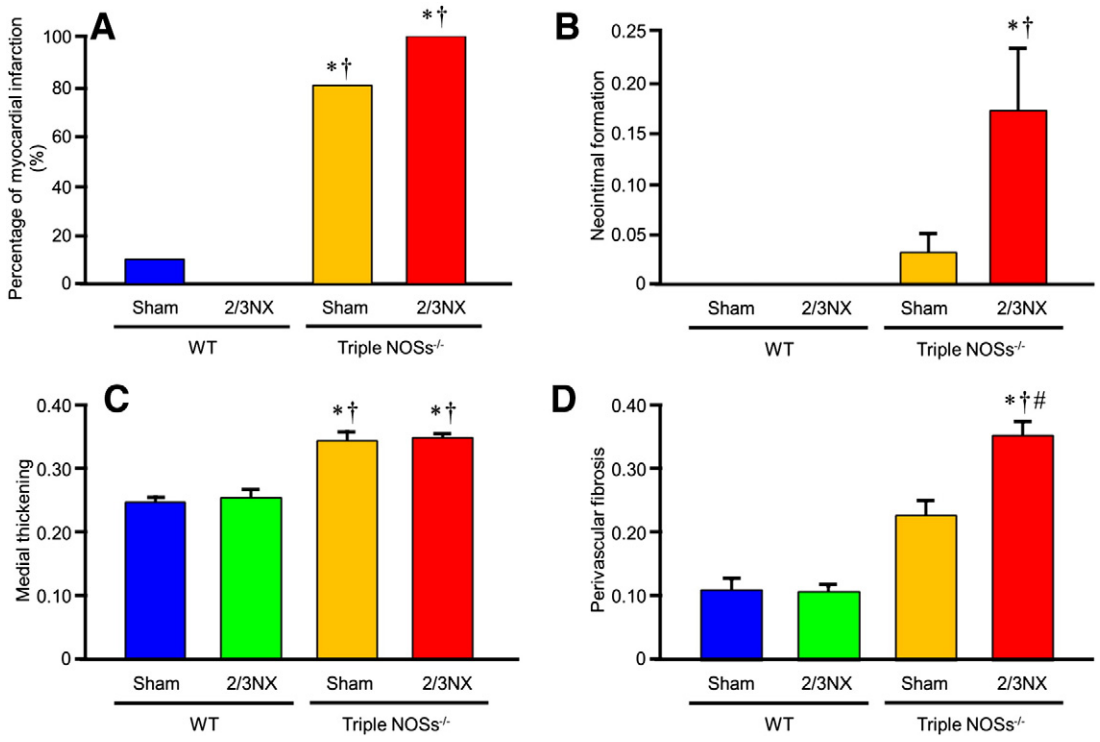


Fig. 4. Coronary arteriosclerotic lesion formation in 2/3NX triple NOSs$^{-/-}$ mice at 2 months after the surgery. After the echocardiography and telemetry ECG, pathological examination of the heart was performed. Four 2/3NX triple NOSs$^{-/-}$ mice that died before 2 months after the surgery and 3 2/3NX triple NOSs$^{-/-}$ mice that died during telemetry ECG were included in the analysis. The heart was cut into 5 equal-thick parts in a short-axis direction, and respective 5 sections were examined. (A) Percentage of acute and/or old myocardial infarction (n = 10–16). NOSs$^{-/-}$, triple NOSs$^{-/-}$ mice; WT, wild-type mice; sham, sham-operated. (B) Neointimal formation (the ratio of intima area to media area) (n = 10–16). (C) Medial thickening (the ratio of media area to total vascular area) (n = 10–16). (D) Perivascular fibrosis (the ratio of perivascular area to total vascular area) (n = 10–16). $^{*}P < 0.05$ vs. sham-operated WT mice; $^{\dagger}P < 0.05$ vs. 2/3NX WT mice; $^{\#}P < 0.05$ vs. sham-operated triple NOSs$^{-/-}$ mice.

in the triple NOSs$^{-/-}$ mice, the 2/3NX significantly and markedly reduced the survival rate compared with sham operation, and, importantly, approximately 90% of the 2/3NX triple NOSs$^{-/-}$ mice suddenly died as early as 4 months after the surgery (Fig. 1A).

We next explored the effect of 2/3NX on the incidence of acute myocardial infarction in the triple NOSs$^{-/-}$ mice by a postmortem examination, which revealed a marked increase in the incidence of myocardial infarction (the percentage of death due to myocardial infarction in the total causes of death) compared with sham operation. Noticeably, 87.8% (43/49) of the 2/3NX triple NOSs$^{-/-}$ mice died due to acute and/or old myocardial infarction (Fig. 1B). It was conceivable that the 2/3NX triple NOSs$^{-/-}$ mice would die mainly due to myocardial infarction-complicated arrhythmias or heart failure (including cardiogenic shock). It is difficult to distinguish between death due to arrhythmias and heart failure since heart failure is often accompanied by arrhythmias and since arrhythmias are always seen prior to any death. Thus, we categorized those causes of death as death due to myocardial infarction. No cerebrovascular disease was observed in any of the dead 2/3NX triple NOSs$^{-/-}$ mice. Fig. 1C represents the lateral wall myocardial infarction seen in the dead 2/3NX triple NOSs$^{-/-}$ mice. The coronary arteries of the dead 2/3NX triple NOSs$^{-/-}$ mice exhibited severe coronary arteriosclerotic lesion formation, including infiltration of inflammatory cells (Fig. 1D), neointimal formation (Fig. 1F), medial thickening (Fig. 1F), perivascular fibrosis (Fig. 1F), and fibrinoid necrosis (Fig. 1D), as well as coronary thrombus formation (Figs. 1E, F). On the other hand, coronary atherosclerotic lesions, such as extracellular lipid accumulation, atheromatous plaque formation, or infiltration of foamy macrophages in the coronary artery, were rarely observed.

3.2. 2/3NX caused echocardiographic and electrocardiographic abnormalities and accelerated coronary arteriosclerotic lesion formation in triple NOSs$^{-/-}$ mice at 2 months after the surgery

We then examined cardiac functional abnormalities and the extent of coronary arteriosclerotic lesion formation in the 2/3NX triple NOSs$^{-/-}$ mice at 2 months post-surgery via echocardiography, telemetry electrocardiography (ECG), and pathological examination. Of the 16 2/3NX triple NOSs$^{-/-}$ mice, 4 died before 2 months after the surgery. Echocardiography showed regional wall motion abnormality in 30% (3/10) of the 2/3NX triple NOSs$^{-/-}$ mice and 10% (1/10) of the sham triple NOSs$^{-/-}$ mice (Fig. 2A). Wall thickness of interventricular septum and posterior wall tended to be thinner and fractional shortening tended to be more reduced in the 2/3NX triple NOSs$^{-/-}$ mice as compared with the sham triple NOSs$^{-/-}$ mice, and fractional shortening was significantly decreased in the 2/3NX triple NOSs$^{-/-}$ mice when compared with the sham WT mice (Figs. 2B, C, E). There was no significant difference in left ventricular end-diastolic dimension between the 2/3NX triple NOSs$^{-/-}$ mice and other mice (Fig. 2D).

Of the 12 2/3NX triple NOSs$^{-/-}$ mice that received subcutaneous implantation of telemetry transmitters, 3 died during ECG recording (within 24 hours after the implantation), and ECG revealed ST-segment elevation followed by sinus arrest, ST-segment elevation followed by advanced atrioventricular block, and ST-segment depression followed by sinus arrest (Fig. 3A). Transient ST-segment depression was detected in other 2 2/3NX triple NOSs$^{-/-}$ mice and 1 sham triple NOSs$^{-/-}$ mice. No ischemic ECG change was seen in sham or 2/3NX WT mice. We evaluated heart rate variability parameters, such as low-frequency (LF) power, high-frequency (HF) power, and LF/HF ratio.

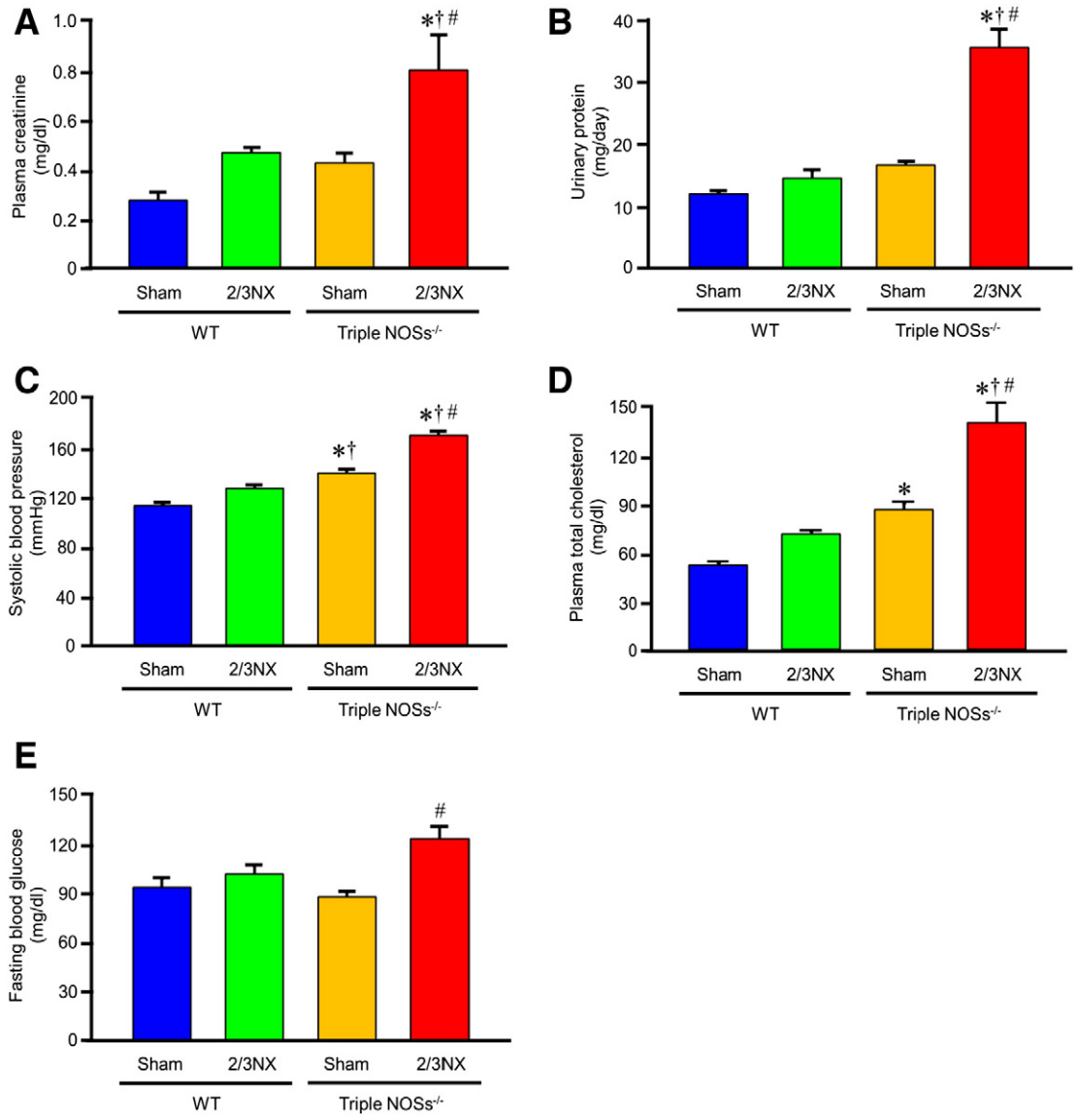


Fig. 5. Renal dysfunction and cardiovascular risk factors in the 2/3NX triple NOSs$^{-/-}$ mice. These parameters were assessed at 2 months after the surgery. (A) Plasma creatinine levels ($n = 10$). (B) Urinary protein levels ($n = 12$). (C) Systolic blood pressure ($n = 12$). (D) Plasma total cholesterol levels ($n = 10$). (E) Fasting blood glucose levels ($n = 10$). $^{*}P < 0.05$ vs. sham-operated WT mice; $^{\dagger}P < 0.05$ vs. 2/3NX WT mice; $^{\#}P < 0.05$ vs. sham-operated triple NOSs$^{-/-}$ mice.

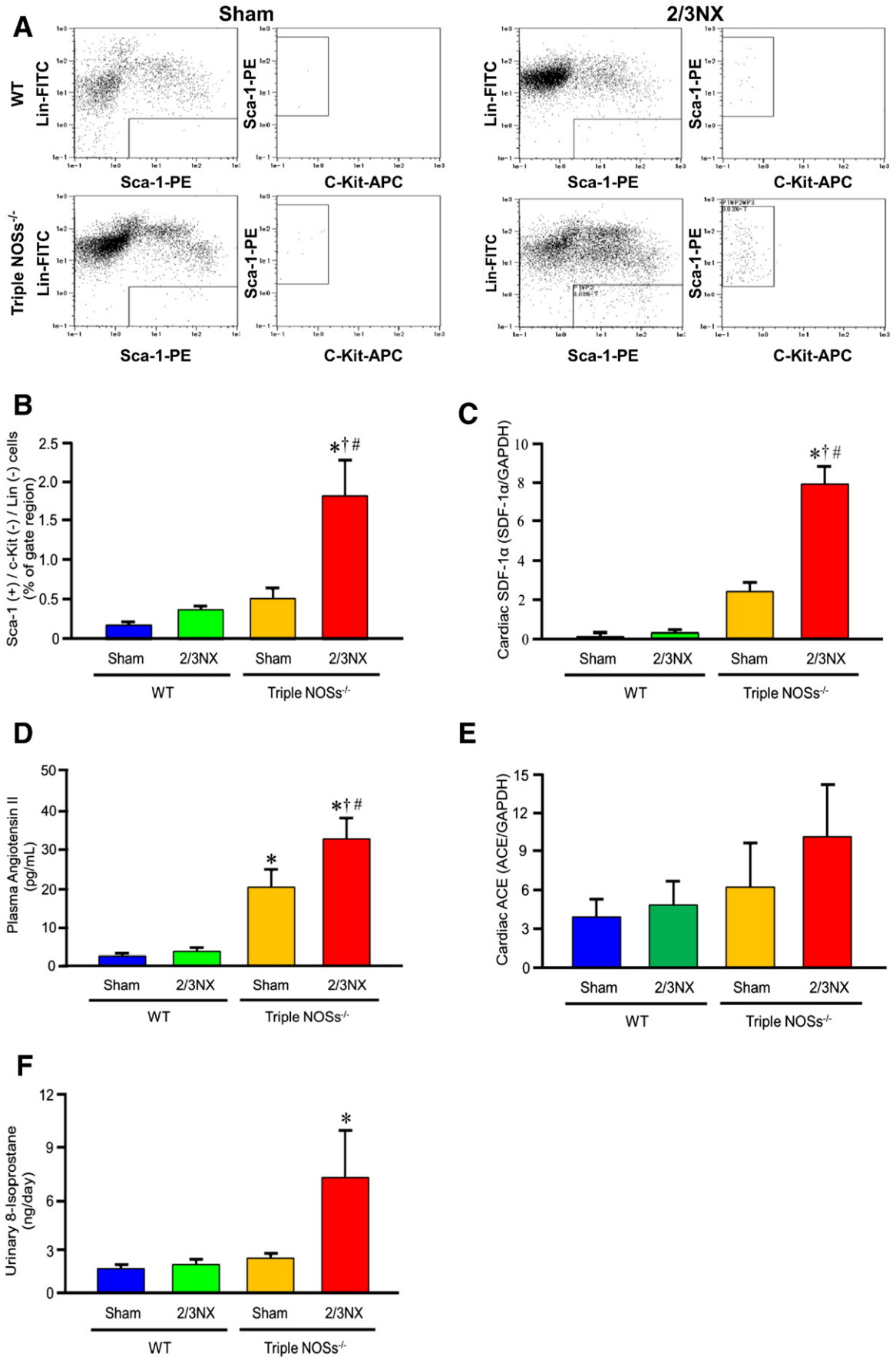
The LF power and the HF power tended to be increased in the 2/3NX triple NOSs$^{-/-}$ mice, and the LF/HF ratio was significantly decreased in the 2/3NX triple NOSs$^{-/-}$ mice as compared with the sham NOSs$^{-/-}$ mice (Figs. 3B–D).

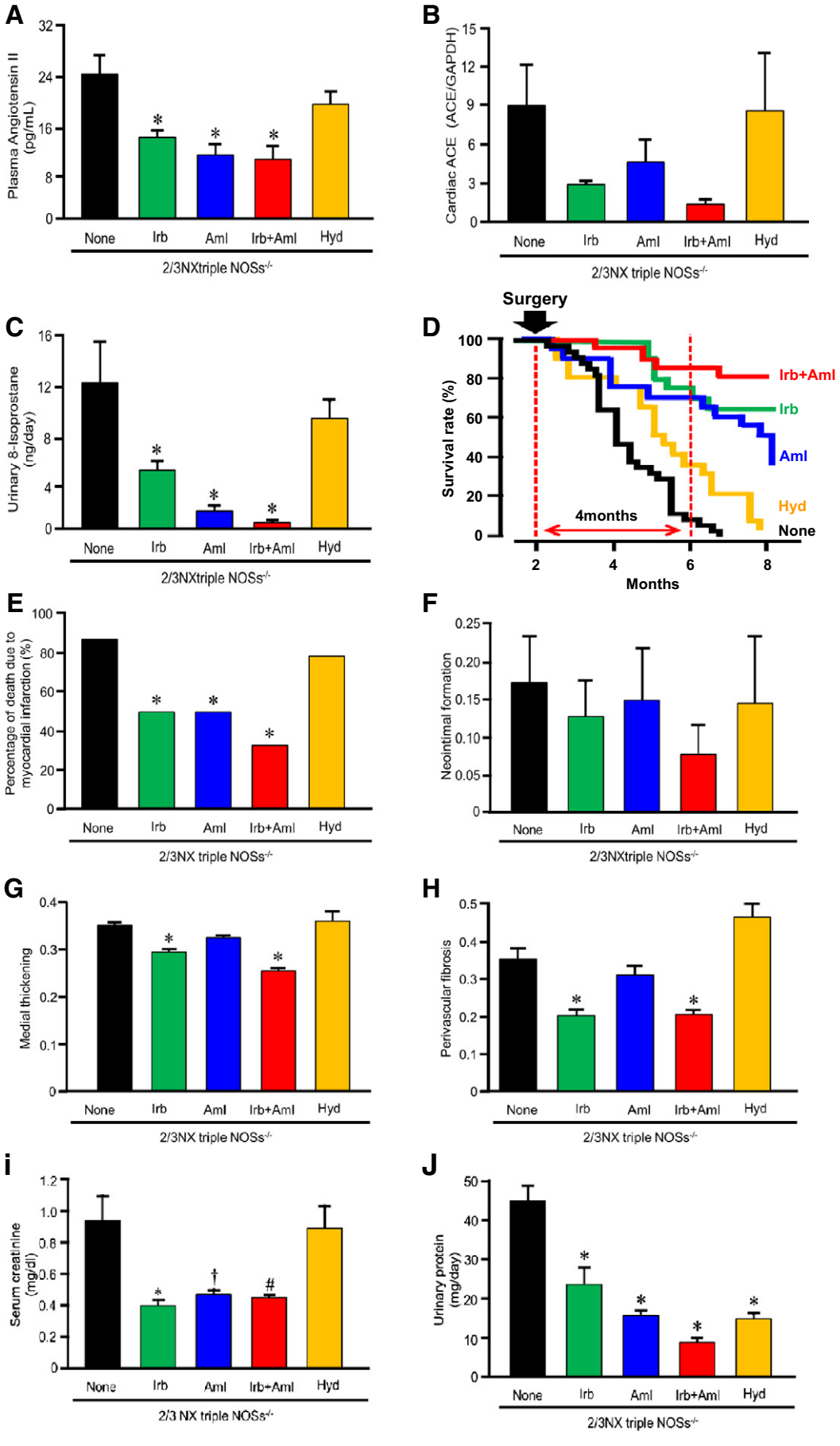
After echocardiography and telemetry ECG, we quantitated the extent of coronary arteriosclerosis. Four 2/3NX triple NOSs$^{-/-}$ mice that died before 2 months after the surgery and 3 2/3NX triple NOSs$^{-/-}$ mice that died during telemetry ECG were included in the analysis. The heart was cut into 5 equal-thick parts in a short-axis direction, and respective 5 sections were examined. Acute and/or old myocardial infarction was recognized in 100% (16/16) of the 2/3NX triple NOSs$^{-/-}$ mice and 80% (8/10) of the sham triple NOSs$^{-/-}$ mice (Fig. 4A). The extents of neointimal formation, medial thickening, and perivascular fibrosis were all markedly accelerated in the 2/3NX triple NOSs$^{-/-}$ mice as compared with the sham WT mice (Figs. 4B–D). Coronary thrombus formation was also noted in 1 2/3NX triple NOSs$^{-/-}$ mice.

3.3. 2/3NX reduced renal function in triple NOSs$^{-/-}$ mice

There were significant increases in plasma creatinine and urinary protein levels, markers of renal function, after the 2/3NX (assessed at 2 months after the surgery) in the triple NOSs$^{-/-}$ mice compared with sham operation (Figs. 5A, B).

Fig. 6. Stromal cell-derived factor (SDF)-1α-induced recruitment of circulating bone marrow-derived vascular smooth muscle cell (VSMC) progenitor cells, renin–angiotensin system activation, and oxidative stress in the 2/3NX triple NOS$^{-/-}$ mice. (A and B) The number of circulating stem cell antigen-1^{+} (Sca-1^{+})/c-Kit^{-}/Lin^{-} cells (interpreted as bone marrow-derived VSMC progenitor cells) analyzed at 1 week after the surgery ($n = 7$). (C) Cardiac SDF-1α protein levels assayed at 1 week after the surgery ($n = 4$–6). (D) Plasma angiotensin II levels measured at 2 months after the surgery ($n = 8$). (E) Cardiac angiotensin-converting enzyme (ACE) protein expression levels evaluated at 2 months after the surgery ($n = 7$). (F) Urinary 8-isoprostane levels assessed at 2 months after the surgery ($n = 8$). $^{*}P < 0.05$ vs. sham-operated WT mice; $^{\dagger}P < 0.05$ vs. 2/3NX WT mice; $^{\#}P < 0.05$ vs. sham-operated triple NOSs$^{-/-}$ mice.


A
Sham
2/3NX
WT
Triple NOSs-/-
Lin-FITC
Sca-1-PE
C-Kit-APC
B
Sca-1 (+) / c-Kit (-) / Lin (-) cells (% of gate region)
C
Cardiac SDF-1α (SDF-1α/GAPDH)
D
Plasma Angiotensin II (pg/mL)
E
Cardiac ACE (ACE/GAPDH)
F
Urinary 8-Isoprostane (ng/day)
Sham
2/3NX
WT
Triple NOSs-/-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Irb
Aml
Irb+Aml
Hyd
2/3NXtriple NOSs-/-
B
Cardiac ACE (ACE/GAPDH)
2/3NX triple NOSs-/-
C
Urinary 8-Isoprostane (ng/day)
D
Surgery
Survival rate (%)
Irb+Aml
Irb
Aml
Hyd
None
4months
Months
E
Percentage of death due to myocardial infarction (%)
F
Neointimal formation
G
Medial thickening
H
Perivascular fibrosis
i
Serum creatinine (mg/dl)
J
Urinary protein (mg/day)

3.4. 2/3NX exacerbated cardiovascular risk factors in triple NOSs$^{-/-}$ mice

Because severe coronary arteriosclerotic lesions were detected in the 2/3NX triple NOSs$^{-/-}$ mice, we then examined the presence or absence of cardiovascular risk factors. The 2/3NX caused significant increases in systolic blood pressure (measured at 1 month after the surgery), plasma total cholesterol levels, and fasting blood glucose levels (evaluated at 2 months after the surgery) in the triple NOSs$^{-/-}$ mice compared with sham operation (Figs. 5C–E).

3.5. 2/3NX caused mobilization of circulating bone marrow-derived vascular smooth muscle cell (VSMC) progenitor cells and up-regulation of cardiac stromal cell-derived factor 1α (SDF-1α) levels in triple NOSs$^{-/-}$ mice

It has been reported that bone marrow-derived VSMC progenitor cells contribute to arteriosclerotic lesion formation after vascular injury and that SDF-1α recruits the VSMC progenitor cells to vascular lesions [19]. We thus analyzed the effects of 2/3NX on the number of circulating bone marrow-derived VSMC progenitor cells and cardiac SDF-1α protein levels in the triple NOSs$^{-/-}$ mice. The 2/3NX significantly and markedly augmented the number of circulating stem cell antigen-1^{+} (Sca-1^{+})/c-Kit^{-}/Lin^{-} cells, which are interpreted as bone marrow-derived VSMC progenitor cells (evaluated at 1 week after the surgery), and the cardiac SDF-1α protein levels (assayed at 1 week after the surgery) in the triple NOSs$^{-/-}$ mice compared with sham operation (Figs. 6A–C and Online Supplementary Fig. I).

3.6. 2/3NX caused renin–angiotensin system activation and oxidative stress in triple NOSs$^{-/-}$ mice

We next investigated the molecular mechanisms for acute myocardial infarction caused by the 2/3NX in the triple NOSs$^{-/-}$ mice. The 2/3NX evoked prominent increases in plasma angiotensin II levels and cardiac angiotensin-converting enzyme (ACE) protein levels, markers of renin–angiotensin system activation (assessed at 2 months after the surgery) in the triple NOSs$^{-/-}$ mice compared with sham operation (Figs. 6D and E, and Online Supplementary Fig. II), although the values of the cardiac ACE protein levels did not reach a statistically significant level because of variations in the data. The 2/3NX also elicited a marked rise in urinary 8-isoprostane levels, a marker of oxidative stress (measured at 2 months after the surgery), in the triple NOSs$^{-/-}$ mice (Fig. 6F).

3.7. Combined treatment with an angiotensin II type 1 (AT_1) receptor blocker, irbesartan, and an antioxidant calcium channel antagonist, amlodipine, markedly prevented coronary arteriosclerotic lesion formation and the occurrence of myocardial infarction and improved the prognosis of 2/3NX triple NOSs$^{-/-}$ mice

Finally, in order to examine the involvement of renin–angiotensin system activation and oxidative stress in the pathogenesis of acute myocardial infarction in the 2/3NX triple NOSs$^{-/-}$ mice, and also in order to validate the experimental usefulness of this acute myocardial infarction model, we investigated the effects on the cardiovascular abnormalities in this model of treatment with a selective and potent AT_1 receptor blocker, irbesartan; an antioxidant dihydropyridine calcium channel antagonist, amlodipine; a combination of both; or an anti-hypertensive agent, hydralazine. We used the clinical therapeutic dosage of irbesartan and amlodipine. Single treatment with irbesartan or amlodipine markedly reduced the plasma angiotensin II levels, the cardiac ACE protein levels, and the urinary 8-isoprostane levels in the 2/3NX triple NOSs$^{-/-}$ mice, while the combined treatment with irbesartan and amlodipine more potently decreased those values (Figs. 7A–C and Online Supplementary Fig. III), although the data of the cardiac ACE protein levels again did not reach a statistically significant level owing to dispersion of the data (Fig. 7B and Online Supplementary Fig. III). Mono-treatment with irbesartan or amlodipine significantly improved the survival rate in the 2/3NX triple NOSs$^{-/-}$ mice, while the irbesartan/amlodipine co-treatment more powerfully ameliorated it. More importantly, these significant effects were noted within the short time of 4 months after the drug treatment, indicating the usefulness of this model for pharmacological studies (Fig. 7D). The sole treatment with irbesartan or amlodipine inhibited the incidence of myocardial infarction (the percentage of death due to myocardial infarction in the total causes of death) and coronary arteriosclerotic lesion formation (neointimal formation, medial thickening, and perivascular fibrosis) in the 2/3NX triple NOSs$^{-/-}$ mice, while the simultaneous treatment with irbesartan and amlodipine more intensely prevented both the incidence of myocardial infarction (Fig. 7E) and coronary lesion formation (Figs. 7F–H). On the other hand, although the treatment with hydralazine significantly lowered systolic blood pressure in the 2/3NX triple NOSs$^{-/-}$ mice to the same extent as the treatment with irbesartan plus amlodipine (Fig. 8A), it did not significantly affect the plasma angiotensin II levels, the cardiac ACE protein levels, the urinary 8-isoprostane levels, the survival rate, the incidence of myocardial infarction, or coronary lesion formation (Figs. 7A–H).

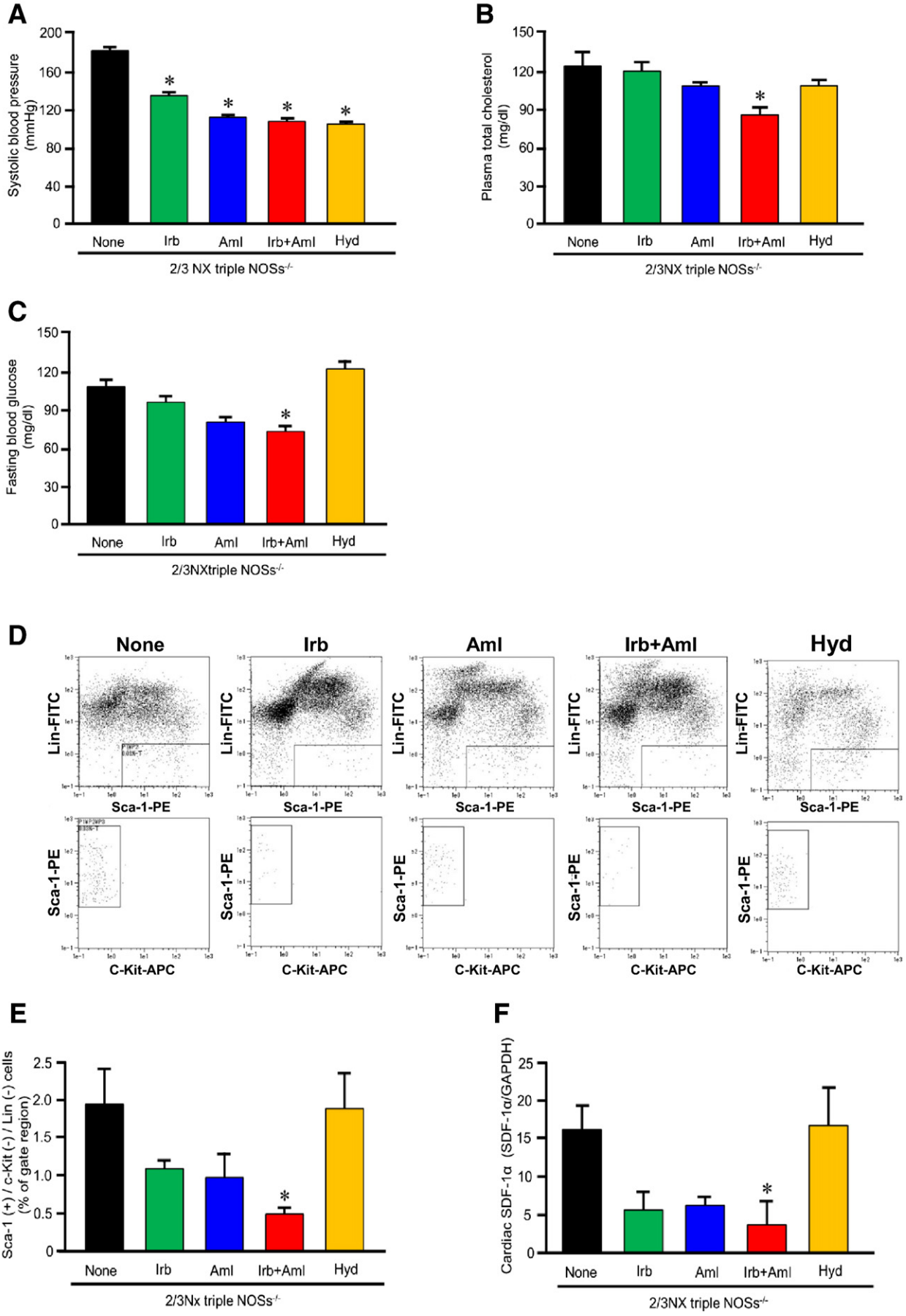
The treatments with irbesartan, amlodipine, and their combination significantly diminished the plasma creatinine levels and the urinary protein levels in the 2/3NX triple NOSs$^{-/-}$ mice (Figs. 7I, J). The treatment with hydralazine also significantly attenuated the urinary protein levels, whereas it had no effect on the plasma creatinine levels (Figs. 7I, J). These results suggest that the decrease in the plasma creatinine levels might have been related to the renal protective actions of the pharmacological agents, while the reduction in the urinary protein levels might have been associated with the lowering of renal intraglomerular pressure induced by these anti-hypertensives.

The plasma total cholesterol levels and the fasting blood glucose levels in the 2/3NX triple NOSs$^{-/-}$ mice tended to be lessened by the treatment with irbesartan or amlodipine, while statistically significant effects were noted only by the combined irbesartan/amlodipine treatment (Figs. 8B, C). Similarly, while the number of circulating Sca-1^{+}/c-Kit^{-}/Lin^{-} cells and the cardiac SDF-1α protein levels in the 2/3NX triple NOSs$^{-/-}$ mice tended to be suppressed by the irbesartan or amlodipine treatment, statistically significant effects were recognized exclusively by the simultaneous treatment with the two agents (Figs. 8D–F and Online Supplementary Fig. IV).

4. Discussion

The major novel findings of the present study are as follows: (i) 2/3NX caused sudden cardiac death due to acute myocardial infarction in male triple NOSs$^{-/-}$ mice as early as 4 months after the surgery. (ii) The 2/3NX triple NOSs$^{-/-}$ mice exhibited electrocardiographic ST-segment elevation, reduced heart rate variability, echocardiographic regional wall motion abnormality, and accelerated coronary

Fig. 7. Effects of treatment with an angiotensin II type 1 (AT1) receptor blocker, irbesartan; an antioxidant calcium channel antagonist, amlodipine; a combination of irbesartan and amlodipine; or an anti-hypertensive agent, hydralazine, on renin–angiotensin system activation, oxidative stress, survival rate, incidence of myocardial infarction, coronary arteriosclerotic lesion formation, and renal function in the 2/3NX triple NOSs$^{-/-}$ mice. Irb, irbesartan (50 mg/kg/day in chow); Aml, amlodipine (3.2 mg/kg/day in drinking water); Hyd, hydralazine (250 mg/mL in drinking water). The effects of the drugs on coronary lesion formation were assessed in the 2/3NX triple NOSs$^{-/-}$ mice at 2 months after the surgery. (A) Plasma angiotensin II levels ($n = 10$). (B) Cardiac ACE protein expression levels ($n = 7$). (C) Urinary 8-isoprostane levels ($n = 8$). (D) Survival rate ($n = 20$–49). (E) Percentage of death due to myocardial infarction in the total causes of death ($n = 6$–49). (F) Neointimal formation (the ratio of intima area to media area) ($n = 6$–16). (G) Medial thickening (the ratio of media area to total vascular area) ($n = 6$–16). (H) Perivascular fibrosis (the ratio of perivascular area to total vascular area) ($n = 6$–16). (I) Serum creatinine levels ($n = 10$). (J) Urinary protein levels ($n = 10$). $^{*}P < 0.05$ vs. none (untreated control).


A
Systolic blood pressure (mmHg)
200
160
120
80
40
0
None
Irb
Aml
Irb+Aml
Hyd
2/3 NX triple NOSs-/-
B
Plasma total cholesterol (mg/dl)
150
120
90
60
30
0
None
Irb
Aml
Irb+Aml
Hyd
2/3NX triple NOSs-/-
C
Fasting blood glucose (mg/dl)
150
120
90
60
30
0
None
Irb
Aml
Irb+Aml
Hyd
2/3NXtriple NOSs-/-
D
None
Irb
Aml
Irb+Aml
Hyd
Lin-FITC
Sca-1-PE
C-Kit-APC
E
Sca-1 (+) / c-Kit (-) / Lin (-) cells (% of gate region)
2.5
2.0
1.5
1.0
0.5
0
None
Irb
Aml
Irb+Aml
Hyd
2/3Nx triple NOSs-/-
F
Cardiac SDF-1α (SDF-1α/GAPDH)
25
20
15
10
5
0
None
Irb
Aml
Irb+Aml
Hyd
2/3NX triple NOSs-/-

arteriosclerotic lesion formation. (iii) Cardiovascular risk factors (hypertension, hypercholesterolemia, and hyperglycemia), an increased number of circulating bone marrow-derived VSMC progenitor cells, and cardiac up-regulation of SDF-1α were noted in the 2/3NX triple NOSs$^{-/-}$ mice and were associated with significant increases in plasma angiotensin II levels and urinary 8-isoprostane levels. (iv) Simultaneous treatment with a clinical dosage of an angiotensin II type 1 receptor blocker, irbesartan, and an antioxidant calcium channel antagonist, amlodipine, markedly prevented coronary arteriosclerotic lesion formation and the incidence of myocardial infarction and improved the prognosis of those mice, along with ameliorating all those pro-arteriosclerotic parameters. Here we report the establishment of a new experimentally useful model of acute myocardial infarction.

4.1. Animal models that develops acute myocardial infarction

Five animal models that emerge acute myocardial infarction have thus far been reported. The first reported acute myocardial infarction model is a rat treated with a non-selective NOS inhibitor, such as N^{ω}-nitro-L-arginine methyl ester (L-NAME) or N^{ω}-nitro-L-arginine (L-NNA), chronically [20–23]. However, we clarified that arteriosclerotic vascular lesion formation caused by long-term treatment with L-NAME or L-NNA is not mediated by simple inhibition of NOSs activities [24]. While L-NAME- or L-NNA-treated rat shows multiple small infarcts without sudden death, those findings are quite different from human pathologies. The L-NAME- or L-NNA-treated rat has not been used at al as an acute myocardial infarction model. The second generated acute myocardial infarction model is the mouse with homozygous null mutations in the genes for both the high-density lipoprotein (HDL) receptor SR-B1 and apolipoprotein (apo) E [25]. The SR-B1$^{-/-}$/apoE$^{-/-}$ mouse dies of acute myocardial infarction before 2 months of age (in childhood) even when fed a standard chow diet [25]. This short-term occurrence of acute myocardial infarction would be useful for experiments. However, the clinical course in human patients with acute myocardial infarction, which usually occurs in adulthood, is different from the natural course in the SR-B1$^{-/-}$/apoE$^{-/-}$ mouse. The third produced model is the myocardial infarction-prone Watanabe heritable hyperlipidemic (WHHLMI) rabbit. The WHHLMI rabbit is not useful for experiments either because it takes a very long time (1 to 3 years) to develop acute myocardial infarction. The fourth created model is the SR-B1$^{-/-}$/hypomorphic apo ER61 (apoER$^{h/h}$) mouse, which shows high-fat diet-induced acute myocardial infarction [26]. Although the SR-B1$^{-/-}$/apoER$^{h/h}$ mouse may be a good model, it has not been used at all in experiments in which the effects of drugs or therapies are examined since its generation was published 9 years ago, and only one article with this mouse has been published after the generation [27]. We reported a fifth model, the triple NOSs$^{-/-}$ mouse, that spontaneously develops acute myocardial infarction. Unfortunately, however, it takes a very long time (approximately 1 year) for acute myocardial infarction to occur in our mouse. In the present study, the majority of the 2/3NX triple NOSs$^{-/-}$ mice exhibited sudden cardiac death due to acute myocardial infarction within as little as 4 months after the surgery, and the experimental usefulness of this model was validated by demonstrating the preventive effects of the combined treatment with irbesartan and amlodipine on the occurrence of acute myocardial infarction. Therefore, our 2/3NX triple NOSs$^{-/-}$ mouse is a new experimentally useful model of acute myocardial infarction.

Severe coronary arteriosclerosis, including infiltration of inflammatory cells, neointimal formation, medial thickening, and perivascular fibrosis, as well as coronary thrombus formation, was noted in the 2/3NX triple NOSs$^{-/-}$ mice. These findings closely resemble the human pathology seen in the infarct-related coronary arteries in patients with myocardial infarction. We previously indicated that endothelium-dependent relaxations to acetylcholine are completely lacking in the triple NOSs$^{-/-}$ mice and that contractions to phenylephrine are markedly enhanced, suggesting the presence of vascular dysfunction in the triple NOSs$^{-/-}$ mice [11]. Thus, it is likely that acute myocardial infarction in the 2/3NX triple NOSs$^{-/-}$ mice resulted from coronary arteriosclerosis, coronary thrombosis, and coronary vasospasm.

Heart rate variability is considered a noninvasive marker to evaluate autonomic nervous system function. It has been reported that low heart rate variability has prognostic value in patients with myocardial infarction and is associated with a higher risk of death in patients with coronary artery disease [28,29]. Consistent with the findings, significantly lower LF/HF ratio was noted in the 2/3NX triple NOSs$^{-/-}$ mice.

4.2. Clinical implications

Several lines of evidence imply the clinical significance of the 2/3NX triple NOSs$^{-/-}$ model. First, the natural course in which acute myocardial infarction occurs in the triple NOSs$^{-/-}$ mice with partial nephrectomy closely resembles the clinical course in which patients with CKD develop acute myocardial infarction. Second, it has been suggested that the defective NOSs system is present in patients with CKD [30], as evidenced by the facts that in such patients urinary NOx excretion, a marker of systemic NO production derived from all three types of NOSs, are reduced [31], that whole body NO production (assessed by giving an intravenous infusion of [$^{15}N_2$]-arginine and measuring isotopic plasma enrichment of [^{15}N]-citrulline) is decreased [32], and that plasma levels of asymmetric dimethylarginine (ADMA), an endogenous NOS inhibitor, are elevated [33]. Finally, it has been reported that the defective NOSs system also exists in patients with coronary arteriosclerosis and myocardial infarction, as demonstrated by the findings that plasma and/or urinary NOx levels are reduced in such patients [34], that plasma ADMA concentrations are elevated in patients with arteriosclerosis and risk of myocardial infarction [35], and that the NOS gene polymorphisms are associated with arteriosclerosis, risk of myocardial infarction, and low plasma NOx levels in humans [36]. Thus, our acute myocardial infarction model may have clinical implications. However, since pathological conditions of the 2/3NX triple NOSs$^{-/-}$ mice may be different from those of the patients with CKD, results obtained from our model must be interpreted with caution.

4.3. Mechanisms for acute myocardial infarction in the 2/3NX triple NOSs$^{-/-}$ mice

Because significant increases in systolic blood pressure, plasma total cholesterol levels, and fasting blood glucose levels were noted in the 2/3NX triple NOSs$^{-/-}$ mice, a clustering of cardiovascular risk factors seems to be involved in the pathogenesis of their acute myocardial infarction. In agreement with this evidence, it has been shown that patients with CKD have a high prevalence of those cardiovascular risk factors, and that those factors are associated with increased risks of acute myocardial infarction and sudden cardiac death [37].

It has recently been reported that bone marrow-derived mononuclear cells differentiate into VSMC progenitor cells, which circulate in

Fig. 8. Effects of treatment with an AT1 receptor blocker, irbesartan; a calcium channel antagonist, amlodipine; a combination of irbesartan and amlodipine; or an anti-hypertensive agent, hydralazine, on cardiovascular risk factors and SDF-1α-induced recruitment of circulating bone marrow-derived VSMC progenitor cells in the 2/3NX triple NOSs$^{-/-}$ mice. (A) Systolic blood pressure ($n = 10$–12). (B) Plasma total cholesterol levels ($n = 10$–12). (C) Fasting blood glucose levels ($n = 10$–12). (D and E) The number of circulating Sca-1^{+}/c-Kit^{-}/Lin^{-} cells ($n = 7$). (F) Cardiac SDF-1α protein levels ($n = 7$). *$P < 0.05$ vs. none (untreated control).

the blood, accumulate in vascular wall, and contribute to vascular lesion formation [38,39]. It has also been shown that the CXC chemokine SDF-1α is a pivotal chemotactic factor of bone marrow-derived VSMC progenitor cells [40]. In the present study, the number of circulating Sca-1^{+}/c-Kit^{-}/Lin^{-} cells (interpreted as bone marrow-derived VSMC progenitor cells) [41] and the cardiac SDF-1α protein levels were markedly increased in the 2/3NX triple NOSs$^{-/-}$ mice. Thus, it is possible that SDF-1α-induced recruitment of the circulating bone marrow-derived VSMC progenitor cells was also involved in the occurrence of acute myocardial infarction in the 2/3NX triple NOSs$^{-/-}$ mice.

Renin–angiotensin system activation (as evidenced by increases in plasma angiotensin II levels and cardiac ACE expression levels) and oxidative stress (as indicated by elevation in urinary 8-isoprostane levels) were noted in the 2/3NX triple NOSs$^{-/-}$ mice. Based on these findings, we used the selective and potent AT1 receptor blocker, irbesartan, and the antioxidant calcium channel antagonist, amlodipine, to further examine the involvement of renin–angiotensin system activation and oxidative stress in the pathogenesis of acute myocardial infarction. It has been indicated that amlodipine is a charged molecule, is highly lipophilic, and has a much higher affinity for lipid-laden cellular membranes than do other calcium channel antagonists, exerting a powerful antioxidant activity, independent of its calcium channel antagonistic action [42]. In the present study, the simultaneous treatment with irbesartan and amlodipine potently suppressed renin–angiotensin system activation and oxidative stress, and markedly prevented coronary arteriosclerotic lesion formation and the incidence of myocardial infarction, and improved the prognosis of the 2/3NX triple NOSs$^{-/-}$ mice. Furthermore, the simultaneous irbesartan/amlodipine treatment significantly ameliorated the cardiovascular risk factors, the increased number of circulating Sca-1^{+}/c-Kit^{-}/Lin^{-} cells, and the enhanced cardiac SDF-1α expression levels in those mice. Therefore, it is conceivable that renin–angiotensin system activation and oxidative stress are involved in the pathogenesis of acute myocardial infarction in the 2/3NX triple NOSs$^{-/-}$ mice. Consistent with these results, it has been reported that renin–angiotensin system activation and oxidative stress are recognized in patients with CKD, and that both factors accelerate arteriosclerotic lesion formation [13].

The treatment with hydralazine exerted an anti-hypertensive action to the same extent as the combined treatment with irbesartan and amlodipine. However, the hydralazine treatment did not show any beneficial effects on the incidence of myocardial infarction, the prognosis, or the pro-arteriosclerotic parameters in the 2/3NX triple NOSs$^{-/-}$ mice. Thus, it is suggested that the beneficial effects of the irbesartan/amlodipine treatment are not caused by changes of blood pressure.

4.4. Clinical perspectives

The mechanism(s) by which CKD is complicated by acute myocardial infarction is not fully understood. Our findings provide novel evidence that the NO/NOSs system plays a pivotal role in the pathogenesis of this reno-cardiac connection. The AT1 receptor blockers and calcium channel antagonists are widely used to treat hypertension in patients with CKD, and the former are also employed to retard the progression of CKD. In the present study, the clinical dosage of irbesartan and amlodipine exhibited cardiovascular and renal protective actions in the 2/3NX triple NOSs$^{-/-}$ mice. These results suggest the therapeutic importance of the AT1 receptor blockers and calcium channel antagonists in preventing complications of acute myocardial infarction in CKD as well as the progression of CKD.

4.5. Conclusions

We have succeeded in developing a novel experimentally useful model of acute myocardial infarction. Renin–angiotensin system activation, oxidative stress, cardiovascular risk factors, and SDF-1α-induced recruitment of circulating bone marrow-derived VSMC progenitor cells appear to be involved in the pathogenesis of acute myocardial infarction in the 2/3NX triple NOSs$^{-/-}$ mice. This model may contribute to the elucidation of the pathogenesis of acute myocardial infarction, and to the research and development of novel therapeutic strategies for preventing this fatal cardiovascular disorder.

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Conflict of interest

We obtained irbesartan and amlodipine from the Sumitomo Dainippon Pharma Co, Japan, and received a research fund and donation from the company.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yjmcc.2014.09.021.

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