Oxidative Stress Induced Ventricular Arrhythmia and Impairment of Cardiac Function in Nos1ap Deleted Mice

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Summary

Genome-wide association study has identified that the genetic variations at NOS1AP (neuronal nitric oxide synthase-1 adaptor protein) were associated with QT interval and sudden cardiac death (SCD). However, the mechanism linking a genetic variant of NOS1AP and SCD is poorly understood. We used Nos1ap knockout mice (Nos1ap−/−) to determine the involvement of Nos1ap in SCD, paying special attention to oxidative stress.

At baseline, a surface electrocardiogram (ECG) and ultrasound echocardiography (UCG) showed no difference between Nos1ap−/− and wild-type (WT) mice. Oxidative stress was induced by a single injection of doxorubicin (Dox, 25 mg/kg). After Dox injection, Nos1ap−/− showed significantly higher mortality than WT (93.3 versus 16.0% at day 14, P < 0.01). ECG showed significantly longer QTc in Nos1ap−/− than WT, and UCG revealed significant reduction of fractional shortening (%FS) only in Nos1ap−/− after Dox injection. Spontaneous ventricular tachyarrhythmias were documented by telemetry recording after Dox injection only in Nos1ap−/−. Ex vivo optical mapping revealed that the action potential duration (APD)90 was prolonged at baseline in Nos1ap−/−, and administration of Dox lengthened APD90 more in Nos1ap−/− than in WT. The expression of Bnap and the H2O2 level were higher in Nos1ap−/− after Dox injection. Nos1ap−/− showed a reduced amplitude of calcium transient in isolated cardiomyocytes after Dox injection. Administration of the antioxidant N-acetyl-L-cysteine significantly reduced mortality of Nos1ap−/− by Dox injection, accompanied by prevention of QT prolongation and a reduction in %FS.

Although Nos1ap−/− mice have apparently normal hearts, oxidative stress evokes ventricular tachyarrhythmia and heart failure, which may cause sudden cardiac death. (Int Heart J 2016; 57: 341-349)

Key words: Sudden cardiac death, Lethal arrhythmia, Heart failure, Doxorubicin

Sudden cardiac death (SCD) is the leading cause of mortality in developed countries, and the incidence of SCD is estimated to be approximately 300,000 cases per year in the United States,11 and 36,000 cases per year in Japan.2 NOS1AP, an adaptor protein of NOS1, also known as CAPON (carboxy-terminal PDZ ligand of nNOS), came under the spotlight by genome-wide association study (GWAS) indicating the strongest association between a single nucleotide polymorphism (SNP) at the NOS1AP gene locus and QT interval in various ethnic populations.3,5,9

Another GWAS showed that a SNP in the NOS1AP region was associated with increased all-cause cardiovascular mortality among users of calcium (Ca2+) channel blockers in a Caucasian population.3,6 Subsequent studies exhibited the association between SNPs at NOS1AP and the risk of SCD.4,8 Notably, in the QTSCD study,9 SNPs at NOS1AP loci showed higher prevalence in SCD patients than SNPs at other ion channel loci implicated for the long QT syndrome, such as SCN5A, KCNQ1, KCNH2, and KCNJ2. However, the mechanism linking the NOS1AP genetic variant and SCD has not yet been adequately clarified.

In the heart, nitric oxide (NO) inhibits L-type Ca2+ channels and stimulates sarcoplasmic reticulum (SR) Ca2+ release via the ryanodine receptor.10 Therefore, NO affects myocardial contractility and cardiac repolarization.11 Among 3 types of NO synthase, Nos1 is localized in sarcoplasmic reticulum.12 Nos1-derived NO modulates cardiac contraction and Ca transients.13 Nos1ap is a cytosolic protein binding to Nos1 via its...
PDZ domain and modulates Nos1 activity. We have demonstrated that the overexpression of Nos1ap in guinea pig ventricular myocytes shortened action potential duration (APD) via reduction in the current through L-type Ca\(^{2+}\) channels (I_{CaL}). On the other hand, it has not been elucidated whether Nos1ap affects cardiac contractility.

Doxorubicin (Dox) is an anthracycline antibiotic, utilized as a broad-spectrum anti-cancer drug. Dox is known to have serious cardiotoxic side effects and induce congestive heart failure. The cardiotoxicity of Dox was reportedly caused by increased oxidative stress, especially in acute Dox treatment. Several experimental models indicated that Dox induced production of reactive oxygen species (ROS) via modifying redox cycling in mitochondria.

NO is a critical modulator of the oxidative stress. Thus, in this paper, we aimed to clarify the involvement of the genetic deletion of Nos1ap in cardiac death, focusing on oxidative stress using an acute Dox administration model.

The major causes of cardiac death are ventricular tachyarrhythmias and heart failure. Since Nos1ap affects QT interval, and Nos1 is involved in Ca transient, both causes could be applicable to cardiac death related with Nos1ap genetic variants.

**METHODS**

**Generation of Nos1ap-deficient mice:** For the targeted deletion of Nos1ap, we designed a construct to introduce a neomycin resistance cassette resulting the deletion of exon 3 of the Nos1ap coding sequence. The linearized vector was electroporated into ES cells; positive cloned were selected by G418 on embryonic fibroblast feeder cells as previously described. BamHI and EcoRI digestions of genomic DNA identified a 5.4-kb recombinant allele and a 11.3-kb wild-type allele, when hybridized with an external probe (Figure 1A). The targeted ES cells were microinjected into C57BL6 blastocysts, and the resulting male chimeras were backcrossed onto C57BL/6 mice. The heterozygous N4 generations were intercrossed to generate Nos1ap-deficient mice. All animal care procedures were approved by the Animal Care and Use Committee of Research Institute, National Center for Global Health and Medicine (Tokyo).

**Mice and pathological animal model:** Wild-type (WT) mice were purchased as control (C57BL/6J, CLEA Japan, Tokyo). All experiments were performed with 8-12 weeks old male mice. All animal experiments were carried out with the approval of the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University.

To induce oxidative stress, we applied Dox (Toronto Research Chemicals, Toronto, Canada). Animals underwent single-dose intraperitoneal injection of Dox (25 mg/kg body weight) or normal saline as a control group, as previously described. An antioxidant agent, N-acetyl-L-cysteine (NAC) (Sigma-Aldrich, St. Louis, MO, USA) was administered (2 g/kg body weight) to a subgroup of mice one hour before injection of Dox or normal saline as previously described.

**Surface electrocardiography (ECG) and ultrasound echocardiography (UCG):** Surface ECG and UCG were performed as described previously. Heart rate and QRS duration were measured. QT interval was defined as the interval between the onset of the QRS complex and the end of the negative component of the T wave. QTc was calculated by the following formula: QTc = QT interval (ms) / √ (RR interval (s) × 10). Left ventricular dimensions in diastolic (LVDd) and systolic (LVDs) phase were measured and fractional shortening (%FS) was

![Figure 1](image-url)
calculated. The ECG and UCG were recorded at baseline and 4 days after Dox or normal saline injection.

**Telemetry ECG recording:** A telemetry ECG transmitter (ETA-F10, DSI, St. Paul, MN, USA) was subcutaneously implanted into the back of the mouse. A telemetry receiver was placed under the cage and the output signal of ECG was digitized with PowerLab (ADInstruments, Dunedin, New Zealand). Ambulatory ECG recording was conducted after recovery from the operation, with or without administration of Dox.

**Optical mapping:** Mice were heparinized with 200 IU of heparin, and anesthetized with pentobarbital sodium (65 mg/kg) intraperitoneally. The excised heart was perfused with Tyrode’s solution (135 NaCl, 5.4 KCl, 1.8 CaCl2, 0.53 MgCl2, 0.33 NaH2PO4, 5.5 D-glucose, 5 HEPES (mM), pH 7.35 ± 0.05, oxygenated with 100% O2), and stained with a voltage sensitive dye, di-4-ANEPPS (WAKO, Osaka, Japan). The membrane potential was recorded with a CMOS camera system (MiCAM Ultima, Brainvision, Tokyo) when the motion artifact was completely suppressed with blebbistatin (Sigma-Aldrich, St. Louis, MO, USA). APD and conduction velocity of the left ventricle was calculated during constant pacing (150 ms cycle length) at the LV apex.

**Ca transient:** Mouse cardiomyocytes were enzymatically isolated (Collagenase type II 25 mg, Protease Sigma type14 3.5 mg, BSA 60 mg) as previously reported. The myocytes were kept at 16°C and were used within 8 hours. Cardiomyocytes were loaded with 2.0 µM Fluo-8AM (AAT Bioquest, Sunnyvale, CA, USA) and incubated at 37°C for 15 minutes. Cardiomyocytes were stimulated by a biphasic pulse of 0.5 Hz, 50% above threshold. Ca²⁺ signals were measured with a fluorescence microscope (BZ-9000, Keyence, Osaka, Japan) at excitation and emission wavelengths of 470 and 535 nm, respectively.

**Quantitative RT-PCR:** The mRNA was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. Quantitative RT-PCR was performed using a High-Capacity cDNA Reverse Transcription Kit, and a 7500 real-time PCR system with TaqMan probe for brain natriuretic peptide (Bnp) (No. 4331182, Thermo Fisher Scientific) and Gapd probe as internal control. Measurements for Nos1, Nos2, and Nos3 in the ventricle were shown no significant change after Dox treatment in both WT and Nos1ap−/− mice: 

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\begin{align*}
\text{APD}_{90} & \text{ (39.8 ± 2.0 versus 38.2% ± 2.0,} \\
\text{P} & = 0.001) \text{ but not in WT (Figure 2D).}
\end{align*}
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We next evaluated APD by optical mapping during constant stimulation (Figure 4D, E). In the baseline condition, Nos1ap−/− showed longer APD measured at 90% repolarization (APD₉₀) than WT did (74.6 ± 2.3 versus 67.6 ± 0.9 ms, P = 0.006). Administration of Dox prolonged APD in both groups.
to a similar degree, resulting in significant prolongation of APD90 after Dox treatment in Nos1ap-/- than in WT (94.8 ± 2.5 versus 86.1 ± 2.2 ms, P = 0.003). The conduction velocity of the left ventricular epicardium was measured during constant pacing from the left ventricular apex, which revealed no difference between WT and Nos1ap-/- at baseline or after Dox treatment (Figure 4F).

Impaired cardiac function after Dox injection in Nos1ap-/- mice:
To clarify the mechanism of reduction in cardiac contraction after administration of Dox in Nos1ap-/- mice, Ca transient was evaluated on isolated cardiomyocytes from adult mouse ventricle (Figure 5A, B). The F/F0 was calculated in 50 isolated cardiomyocytes per mouse. At baseline, peak F/F0 showed no significant difference. Administration of Dox reduced peak F/F0 significantly in Nos1ap-/- (1.97 ± 0.04 versus 2.20 ± 0.08, P = 0.002). However, WT mice showed no significant change in peak F/F0 between the Dox and control groups (2.16 ± 0.04 versus 2.22 ± 0.06, P = 0.369). Decay of Ca transient was prolonged after administration of Dox in both WT and Nos1ap-/-, but there was no significant difference between them (Figure 5C).

The expression of Bnp in the left ventricle was significantly increased at 4 days after injection of Dox in Nos1ap-/- mice (Figure 5D). These findings indicated that administration of Dox reduced Ca transient only in Nos1ap-/- mice in the acute oxidative stress model, without a significant influence on the reuptake of [Ca²⁺], resulting in reduced systolic function and heart failure.

We also performed histological evaluation including detection of fibrosis before and 4 days after injection of Dox. However, no fibrotic changes were observed in either WT or Nos1ap-/- by histological analyses, and administration of Dox did not increase the fibrotic area in either group at this time point (Figure 6A, B).

Increased oxidative stress in Nos1ap-/- mice: To further evaluate the mechanisms underlying the development of ventricular arrhythmias and heart failure, we measured H₂O₂ activity before and after Dox treatment (Figure 7A). At baseline, H₂O₂ production was higher in Nos1ap-/- than in WT (22.5 ± 1.9 versus 16.9 ± 0.7 µM, P < 0.001). After Dox treatment, while H₂O₂ production was significantly increased in both groups, the H₂O₂ level was higher in Nos1ap-/- than in WT (39.5 ± 1.7 versus 32.3 ± 1.4 µM, P < 0.001).

If oxidative stress plays a major role in cardiac death in Nos1ap-/-, application of an antioxidant may rescue it. Thus, we administered the antioxidant N-acetyl-L-cysteine (NAC)
Figure 4. Electrophysiological changes after Dox injection. A: Representative ECG trace of ventricular tachyarrhythmias recorded with a telemetry device. Upper panel shows sustained VT (cycle length 74 ms), and lower panel shows VF. Out of 7 mice examined, VT was observed in one mouse, and VF in 2 mice. B: Incidence of ventricular tachyarrhythmias (VT and VF) within 7 days after injection of Dox in WT (n = 16) and Nos1ap–/– (n = 7). C: Averaged prevalence of PVCs at 4 days after Dox injection. D: Representative trace of optical mapping. Using fluorescent intensity of di-4-ANEPPS, membrane potentials of isolated hearts were measured on a Langendorff apparatus. E: APD90 was calculated in saline or Dox injected group in WT or Nos1ap–/– (n = 5-10). F: Conduction velocity of left ventricle calculated in optical mapping. Ctrl indicates saline injected group as a control; VT, ventricular tachycardia;VF, ventricular fibrillation; PVC, premature ventricular contraction; and APD, action potential duration.

Figure 5. Effect of Dox treatment on cardiac function. A: Representative traces of Ca transient in isolated adult cardiomyocytes. By using fluo-8, [Ca2+]i was measured during electrical stimulation at 0.5Hz. B: Peak F/F0 was compared among WT and Nos1ap–/– with administration of Dox or saline (n = 3-4). C: Time from peak to 25% or 75% decay in WT and Nos1ap–/– with and without Dox application. D: mRNA quantification of Bnp (n = 7-11). Bnp expression was normalized by Gapd. Ctrl indicates saline injected group as a control.
before injection of Dox. Pretreatment with NAC significantly reduced the mortality rate in \( \text{Nos1ap}^-\), while there was no improvement in the survival rate in WT (Figure 7B).

To gain an insight into whether NAC improved the survival rate by normalizing Dox-induced changes in electrophysiological characteristics, contractility, or both, we analyzed Dox-treated mice with or without NAC pretreatment. NAC cancelled the electrophysiological changes induced by administration of Dox, including prolongation of the QRS duration and QT interval (Figure 8A). NAC also rescued the reduced %FS evoked by Dox injection (Figure 8B). The frequency of PVCs was also significantly reduced in Dox-injected \( \text{Nos1ap}^-\) by pretreatment with NAC (Figure 8C). Optical mapping demonstrated that NAC shortened the prolonged APD_{90} in both the WT and \( \text{Nos1ap}^-\) groups. Of note, the differences in APD_{90} before and after Dox application between WT and \( \text{Nos1ap}^-\) were no longer present in NAC-pretreated mice (Figure 8D). The conduction velocity of the left ventricle showed no change by pretreatment with NAC in both WT and \( \text{Nos1ap}^-\) (Figure 8E).

**Discussion**

To the best of our knowledge, this paper is the first to describe the involvement of Nos1ap in SCD utilizing a genetica-
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**Figure 8.** Effect of NAC pretreatment after Dox injection. ECG parameters (A) and fractional shortening by UCG (B) before and after administration of Dox with NAC pretreatment (\(n = 6\), each). Pretreatment with NAC canceled the effect by administration of Dox in each parameter. C: PVCs were counted after Dox with or without NAC pretreatment in NOS1AP\(^{-/-}\) group. D: APD\(_{90}\) and conduction velocity were calculated after each pharmacological treatment in WT and NOS1AP\(^{-/-}\) groups. All abbreviations are same as Figures 2 and 4.

Our results indicated that SCD could be caused by electrophysiological dysfunction resulting in lethal ventricular tachyarrhythmias, in addition to impaired cardiac function via reduced Ca\(^{2+}\) release from sarcoplasmic reticulum.

Regarding the role of Nos1ap, Jaffrey, et al have reported that Nos1ap inhibits Ca\(^{2+}\) influx via the NMDA receptor in neurons.\(^{28}\) We previously demonstrated that overexpression of Nos1ap reduced IC\(_{aL}\), resulting in shortening of APD in ventricular myocytes.\(^{14}\) In the present study, we demonstrated Nos1ap regulated Ca\(^{2+}\) release from sarcoplasmic reticulum in addition to the effect on APD. Beigi, et al have also reported that the macromolecular complex including Nos1 and Nos1ap is located in the sarcoplasmic reticulum in cardiomyocytes,\(^{29}\) which is in line with our findings of reduced Ca\(^{2+}\) release from the sarcoplasmic reticulum in this study.

In this study, Nos1ap\(^{-/-}\) did not show any changes in surface ECG and UCG in the baseline condition. It is well known that the measurement of QT interval is difficult in murine ECG due to the lack of an ST portion and obvious terminal of the T wave.\(^{30}\) However, optical mapping revealed that Nos1ap\(^{-/-}\) hearts exhibited significantly prolonged APD. This result is consistent with our previous report that the overexpression of Nos1ap shortened APD in guinea pig ventricular myocytes.\(^{14}\) It could also explain the association of QT interval and SNPs at Nos1ap in several GWAS.\(^{9,31}\)

Surface ECG revealed prolongation in the QRS duration after administration of Dox. However, ex vivo optical mapping failed to show conduction delay. Surface ECG was recorded during spontaneous beating, but the measurement of conduction by optical mapping was performed during pacing from the left ventricular apex. In addition, ex vivo experiments were performed in isolated hearts without autonomic nervous regulation. We believe the discrepancy between in vivo and ex vivo observations is caused by these different experimental conditions. We also observed obvious QRS prolongation just before spontaneous VF by telemetry recording (Figure 4A). Since VF occurred 7 days after injection of Dox, the optical mapping experiments may be too early to delineate the conduction disturbance evoked by Dox. Therefore, we believe the arrhythmogenicity in this model may have been caused by both factors: the prolonged action potential duration and the conduction disturbance.

It was reported that NOS1AP stabilized NOS1 and enhances NOS1-derived NO production.\(^{14}\) Regarding other subtypes of NOS, previous studies have revealed that NOS1AP did not interact with NOS3 in cardiomyocytes,\(^{14}\) or with NOS2 or 3 in the central nervous system.\(^{28}\) We measured the expression of mRNA of Nos1-3, which showed the expression level of all NOS was not different between WT and Nos1ap\(^{-/-}\). The knockout of Nos1ap did not affect the expression of Nos in the mRNA level.

Based on the ambulatory ECG recordings that revealed lethal ventricular tachyarrhythmias, the cause of death in Dox-treated Nos1ap\(^{-/-}\) mice was at least partly due to lethal tachyarrhythmias. APD of Nos1ap\(^{-/-}\) mice was prominently prolonged after Dox injection. APD prolongation may cause ventricular tachyarrhythmias resulting in SCD, probably with conduction disturbance. Thus, in humans, prolongation of the QT interval by NOS1AP polymorphisms may represent the risk of SCD.
On the other hand, we failed to induce ventricular tachyarrhythmia by programmed stimulation in Nos1ap-/- with or without Dox treatment. Ambulatory ECG showed 2 of 3 episodes of ventricular tachyarrhythmias occurred at night, a physically active phase of mice. However, we performed arrhythmia induction studies under anesthesia without application of β-stimulants, which may explain the lack of arrhythmia induction.

As far as we know, cardiac function has not been assessed in the context of Nos1ap. This study is the first to show that the deletion of Nos1ap reduces systolic cardiac function accompanied with reduced Ca transient under oxidative stress and that the macromolecular complex consisting of Nos1 and Nos1ap regulates the opening of the ryanodine receptor, and the deletion of Nos1ap impairs its function. We have also observed that the decay of \([\text{Ca}^{2+}]\) slows after administration of Dox in both WT and Nos1ap-/-. It is reported that Dox inhibited the phosphorylation of phospholamban, which resulted in decreased calcium uptake by sarcoplasmic reticulum.\(^6\) Since the degree of slowing of decay in \([\text{Ca}^{2+}]\), was similar between WT and Nos1ap-/-, the slowed decay was caused by the direct effect of Dox, rather than the involvement of Nos1ap.

The measurement of H2O2 in the baseline state showed a subclinical but significant increase in oxidative stress in Nos1ap-/- hearts. Administration of Dox enhanced the oxidative stress, which manifested the phenotype in Nos1ap-/-. We utilized a single-dose injection model of Dox. This model did not induce fibrosis or dilatation of the left ventricle, compared with the Dox-induced cardiomyopathy model with repeated administration of Dox for several weeks.\(^7\) Thus, this single dose injection model is rather specific as an oxidative stress model.

Since the increased mortality rate in Nos1ap-/- was cancelled by pretreatment with NAC, oxidative stress was the major cause of death in Nos1ap-/- after Dox injection. A previous study indicated that the oxidative stress induced by \(\text{H}_{2}\text{O}_2\) prolonged APD accompanied by early after depolarization in ventricular myocytes.\(^8\) However, some of the animals died after administration of Dox even with NAC, as reported previously.\(^9\) Taken together, it is possible that the pretreatment with NAC could not protect against oxidative stress completely, or that direct DNA/RNA damage contributed to the mortality. Since NAC did not improve the mortality of WT with Dox administration, DNA/RNA damage or mitochondrial toxicity might have contributed to the death in Dox-injected WT mice. The doxorubicin model in this paper was an acute model due to the single injection. Thus, we cannot simply expand our results to a chronic doxorubicin model or clinical doxorubicin-induced cardiomyopathy regarding anti-oxidative therapy. **Conclusion:** Genetic deletion of Nos1ap evoked prolonged APD and enhanced oxidative stress at a subclinical level. Administration of Dox enhanced oxidative stress, resulting in prolonged QTc, lethal ventricular arrhythmias, and impaired cardiac function in Nos1ap-/- mice. Both the electrophysiologically changes and impaired systolic function contributed to the increased mortality in Nos1ap-/- under oxidative stress.

**REFERENCES**