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(54) **INHIBITION OF OXIDATIVE STRESS IN
ATRIAL FIBRILLATION**

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A61B 5/00 (2006.01)

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(52) **U.S. Cl.**
CPC *C12N 15/1137* (2013.01); *C12Y 106/03* (2013.01); *C12N 2310/14* (2013.01); *A61B 5/046* (2013.01); *A61B 5/4848* (2013.01); *A61B 5/04012* (2013.01); *C12N 2310/531* (2013.01)

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(21) Appl. No.: **15/943,069**

(22) Filed: **Apr. 2, 2018**

(57) **ABSTRACT**

Related U.S. Application Data

(62) Division of application No. 14/542,501, filed on Nov. 14, 2014, now Pat. No. 9,932,588.

Disclosed herein are pharmaceutical compositions and methods for inhibiting oxidative stress in a subject having atrial or ventricular arrhythmias, ventricular failure or heart failure. The methods include administering an effective amount of a NOX2 inhibitor agent to the subject, wherein said administering is under conditions such that a level of oxidative stress in myocardial tissue is reduced or eliminated. The pharmaceutical compositions include a NOX2 inhibitor agent.

(60) Provisional application No. 61/904,925, filed on Nov. 15, 2013.

Specification includes a Sequence Listing.

Publication Classification

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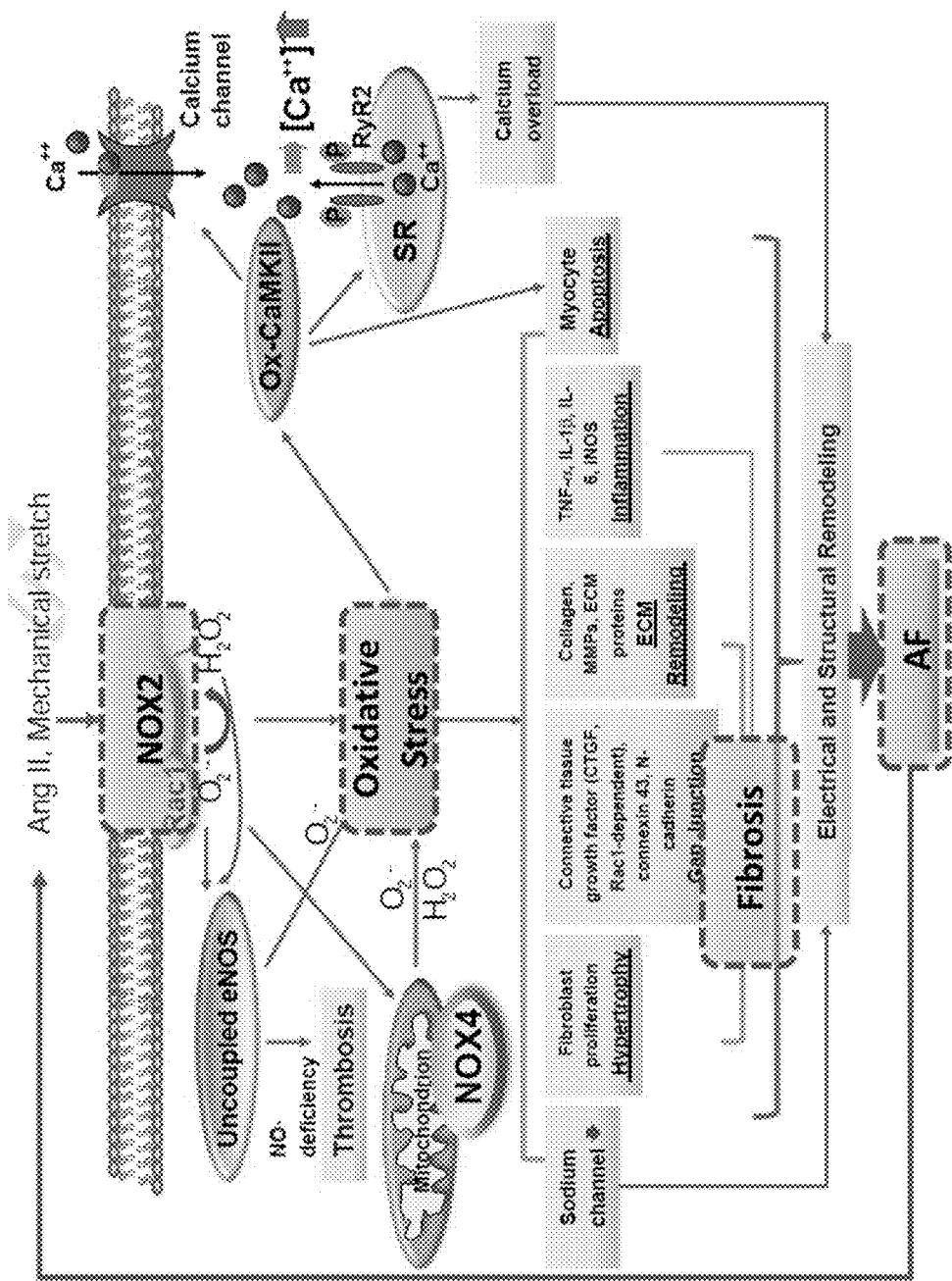


FIG. 1

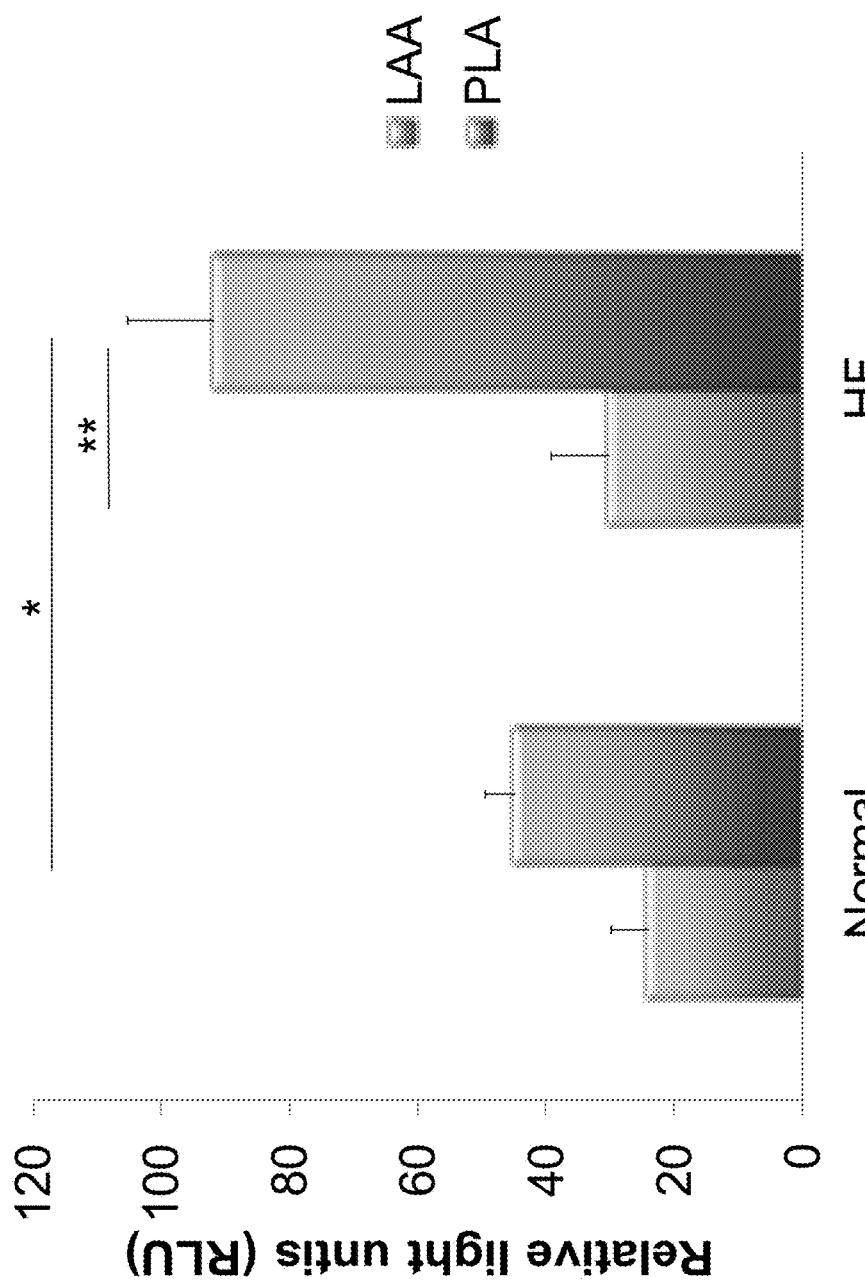


FIG. 2A

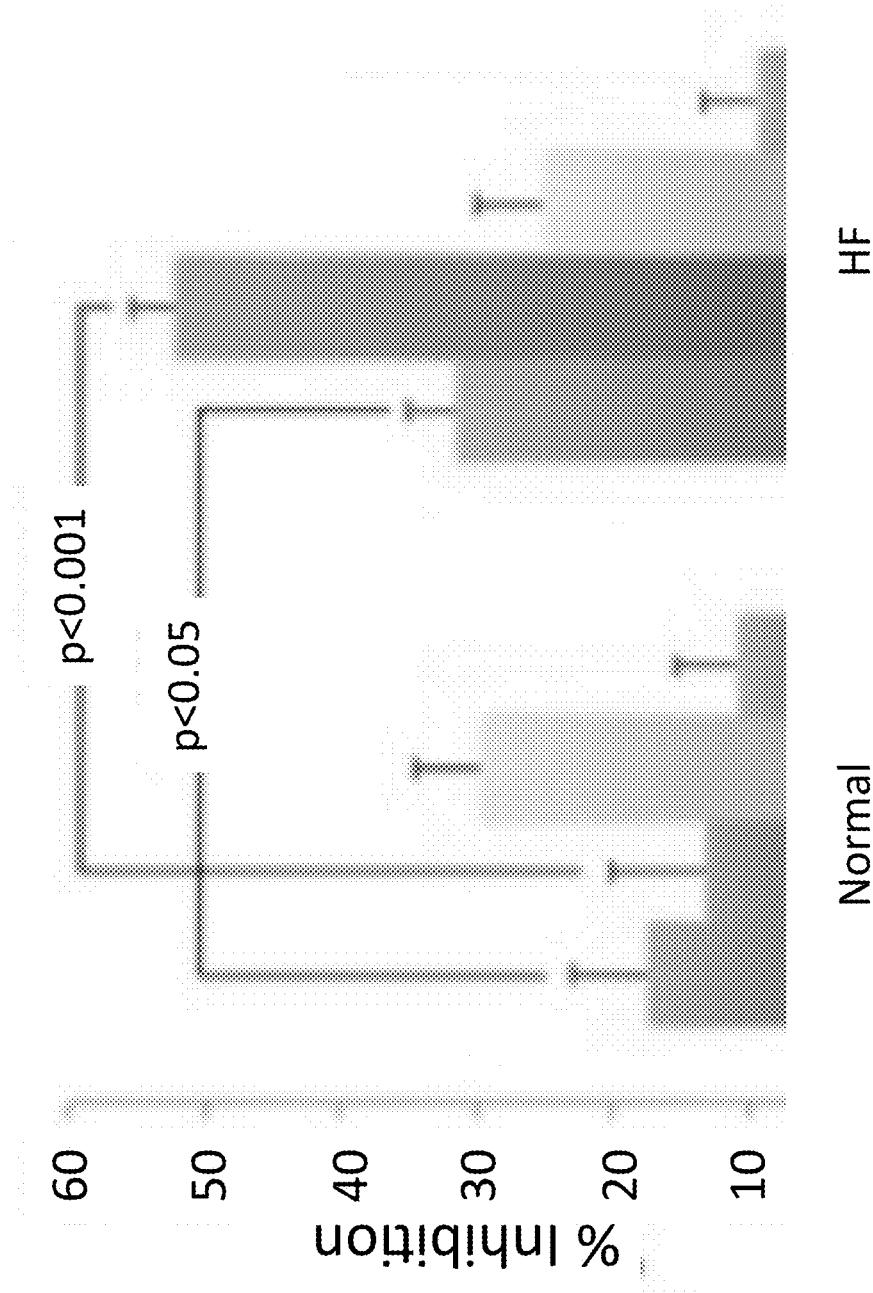


FIG. 2B

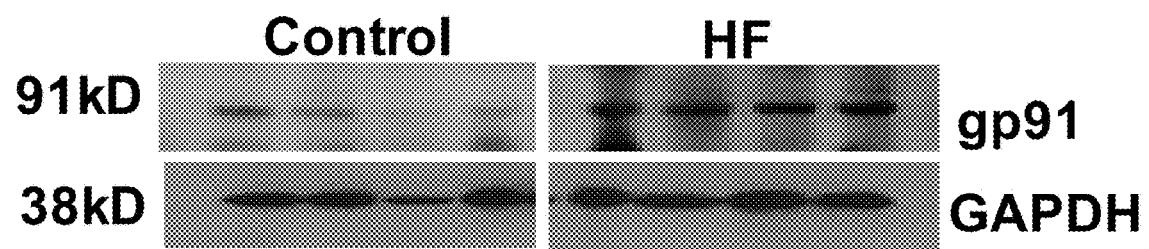


FIG. 3A

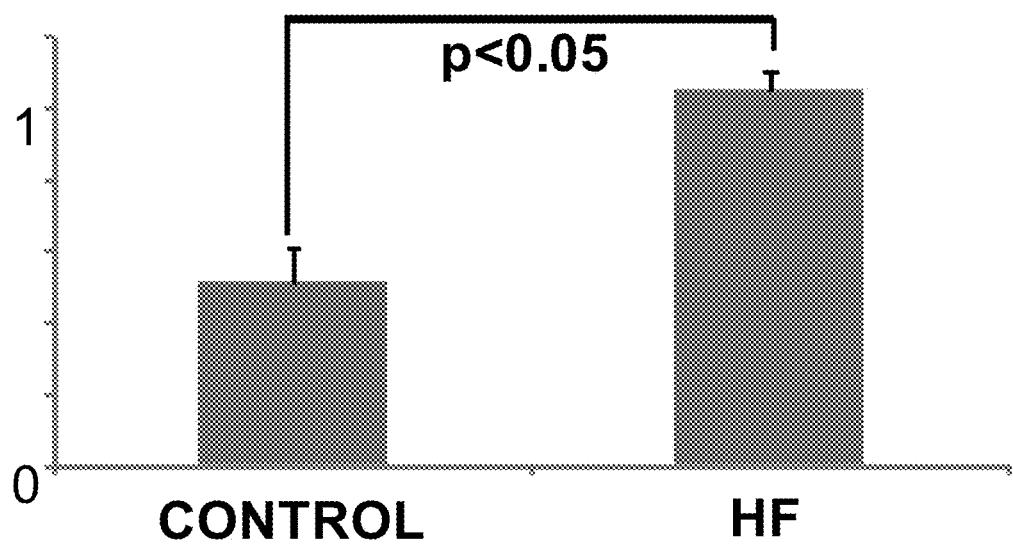


FIG. 3B

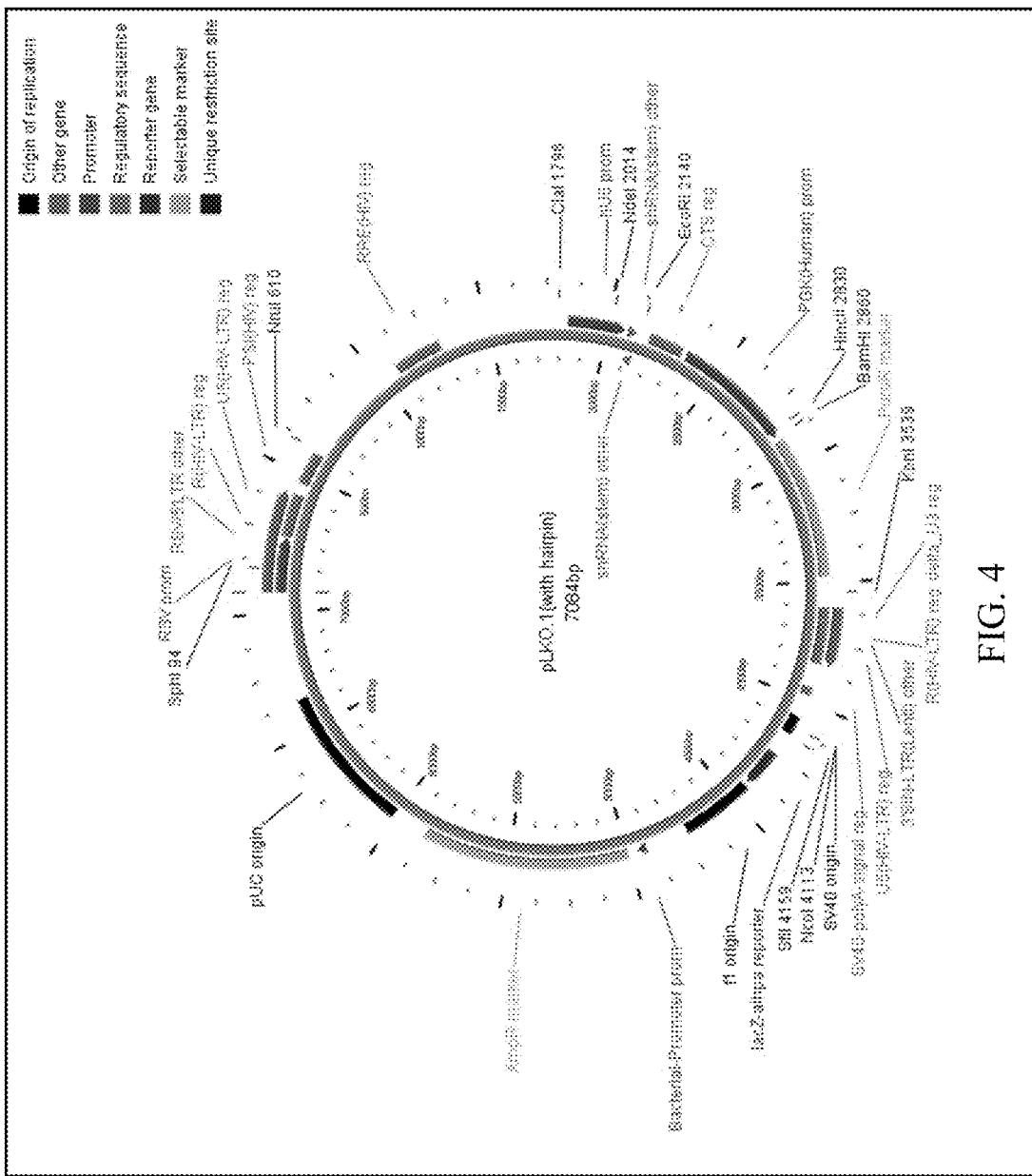


FIG. 4

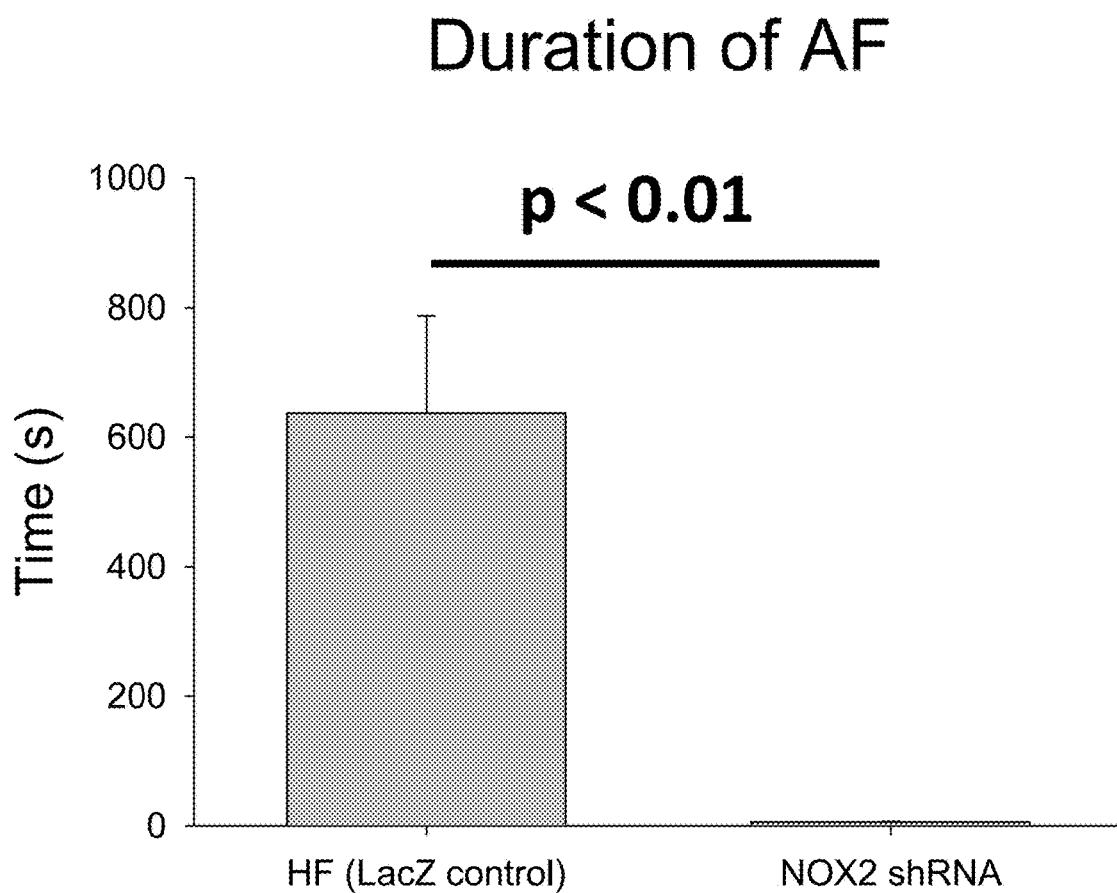


FIG. 5

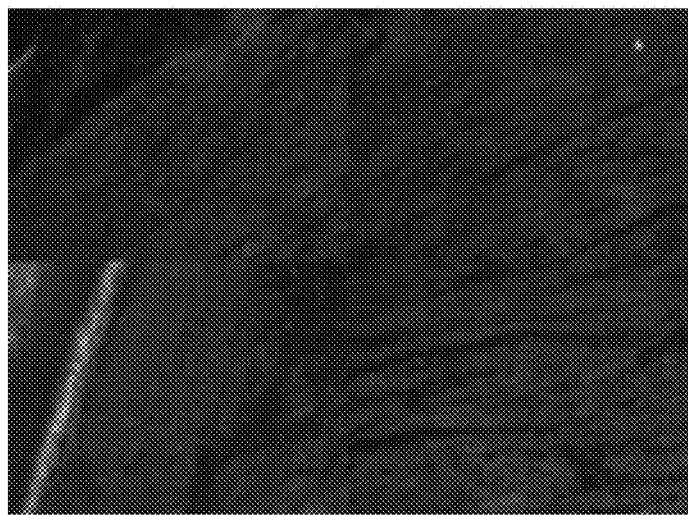


FIG. 6A

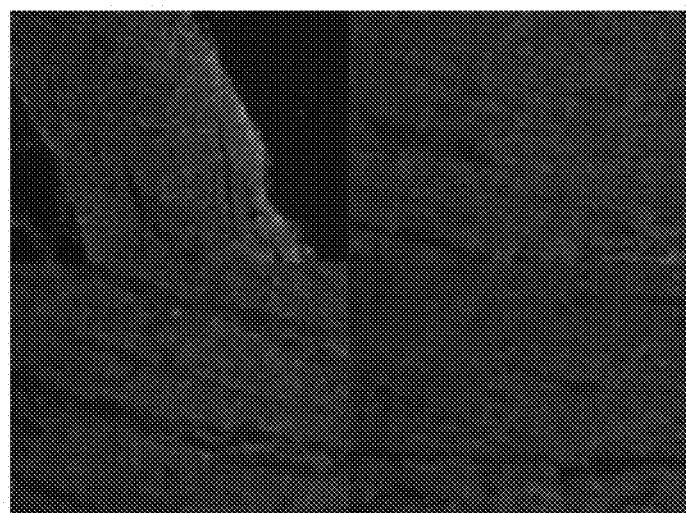


FIG. 6B

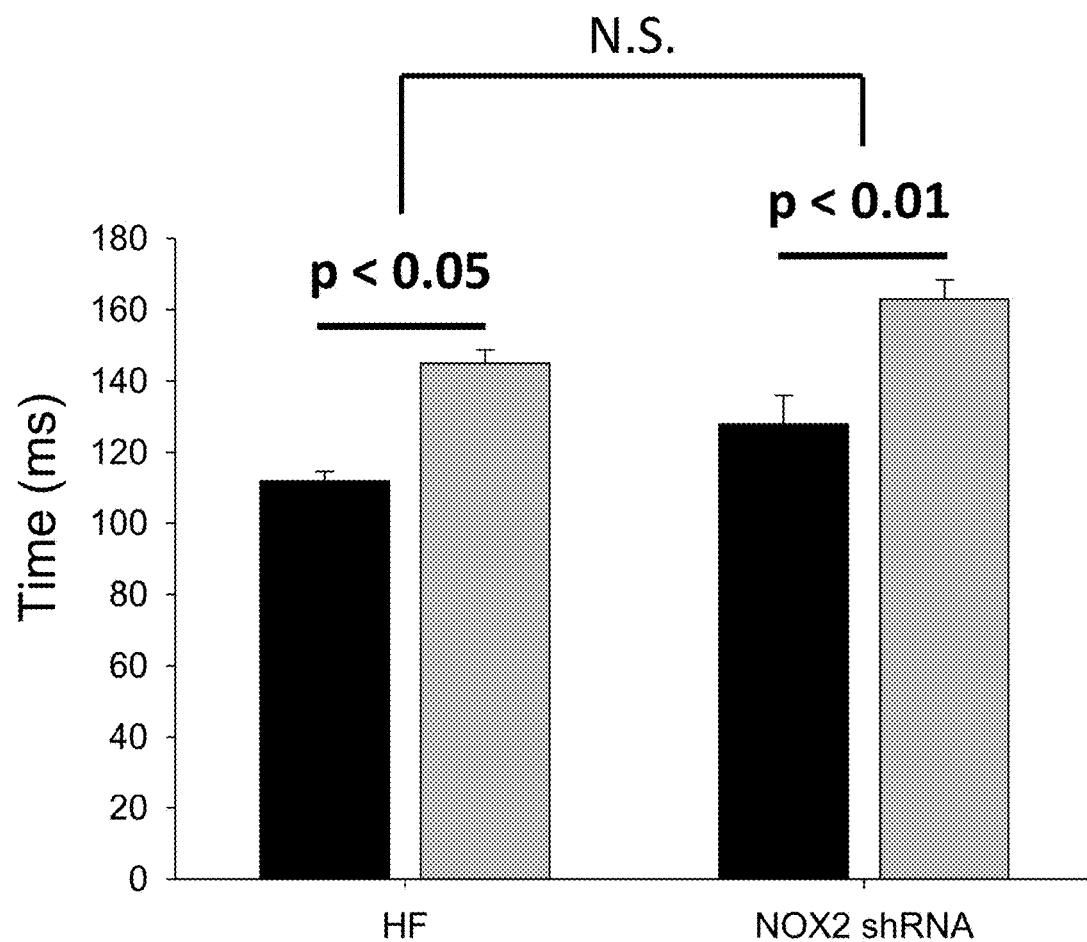


FIG. 7

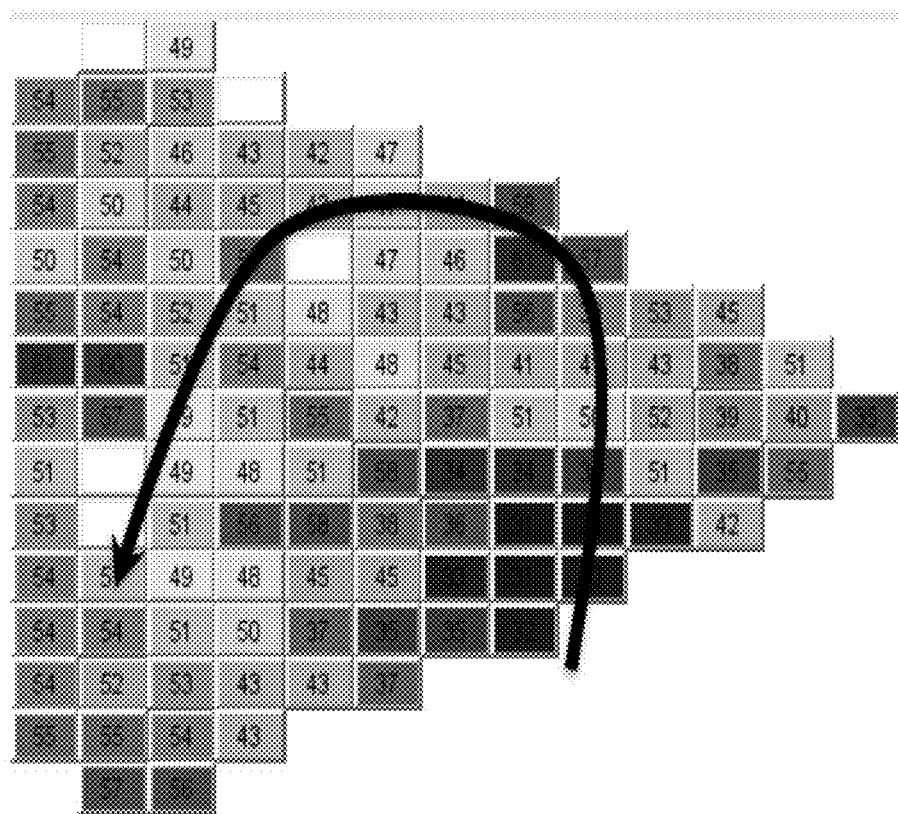


FIG. 8A

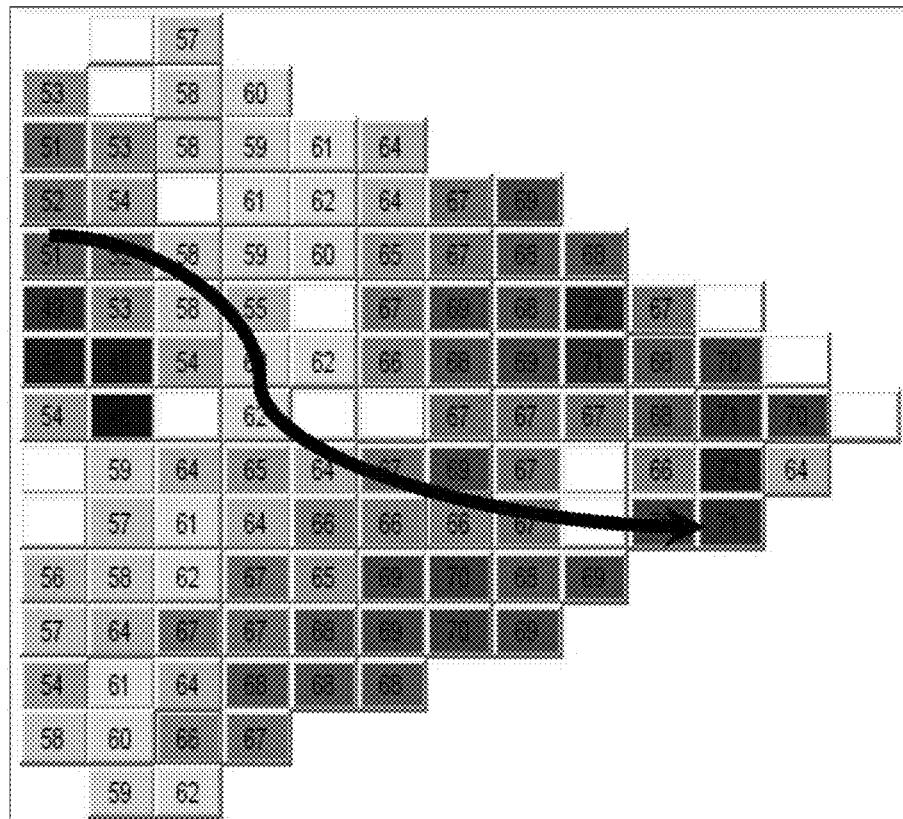


FIG. 8B

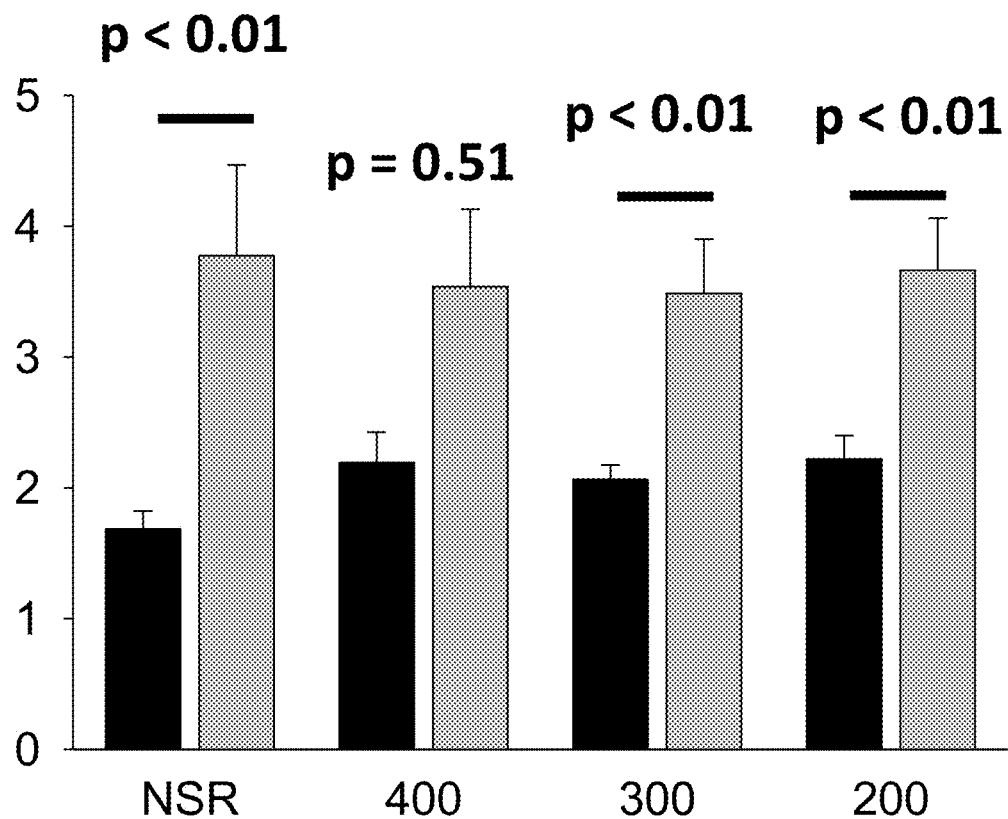


FIG. 8C

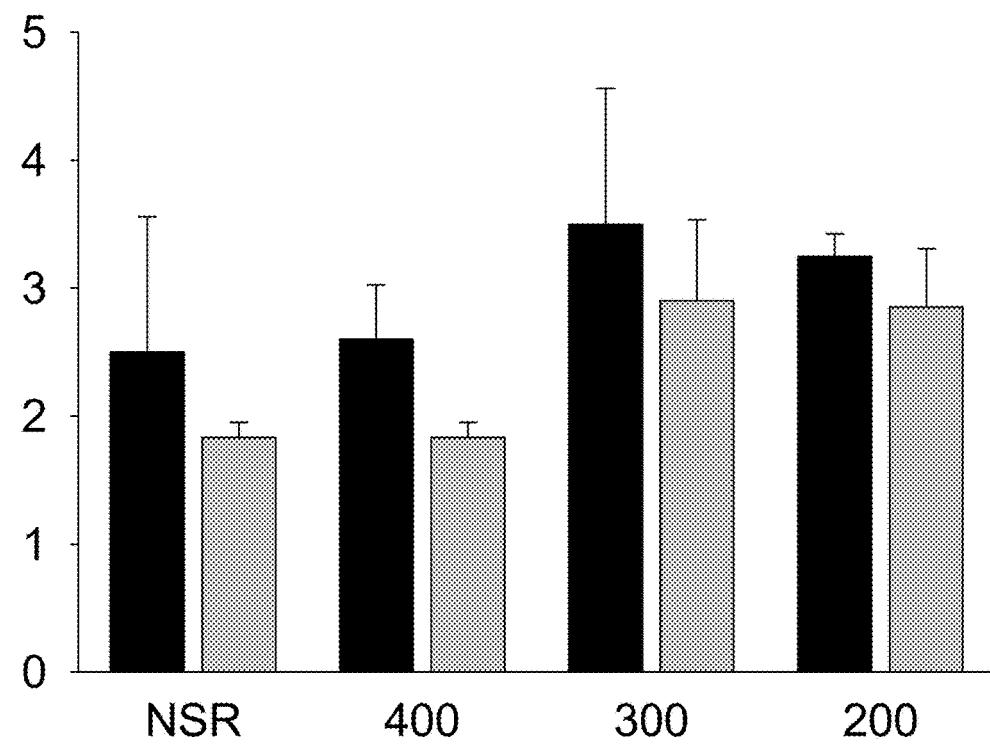


FIG. 8D

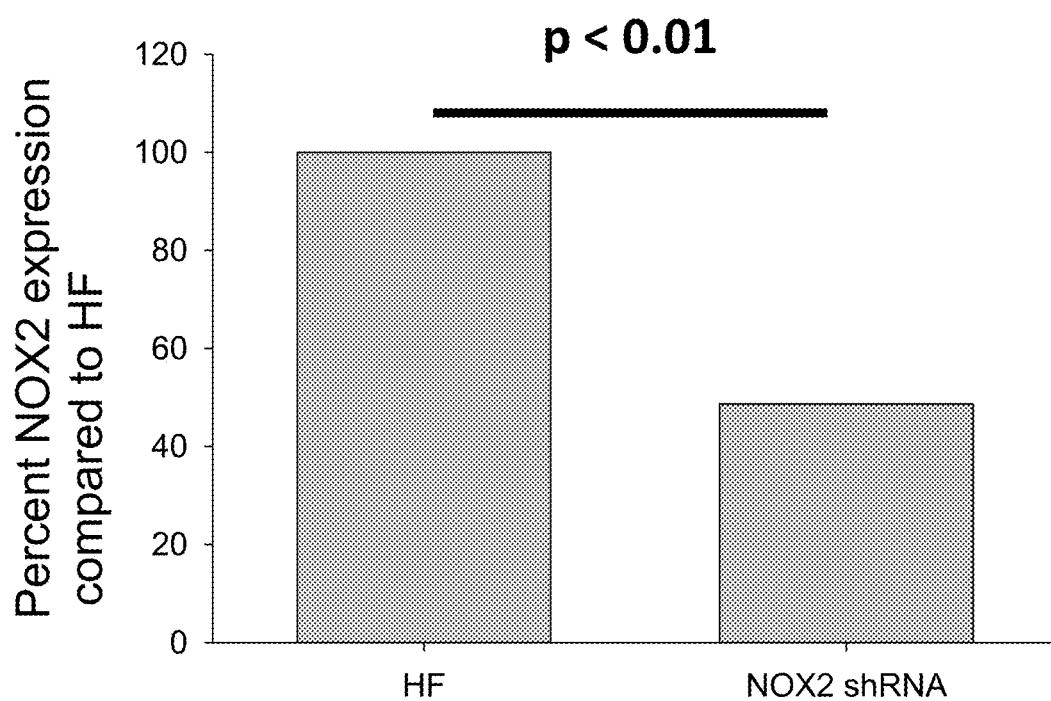


FIG. 9A

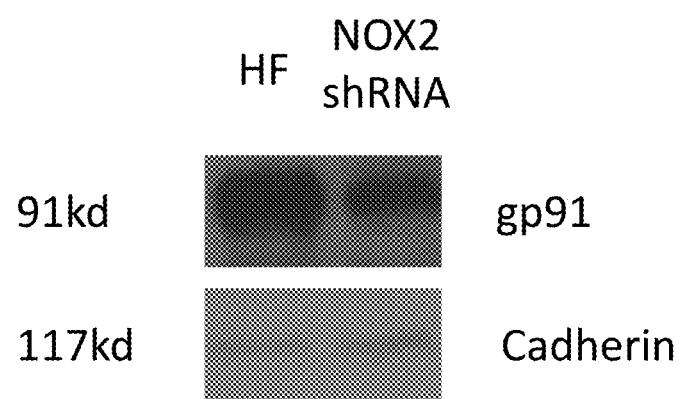


FIG. 9B

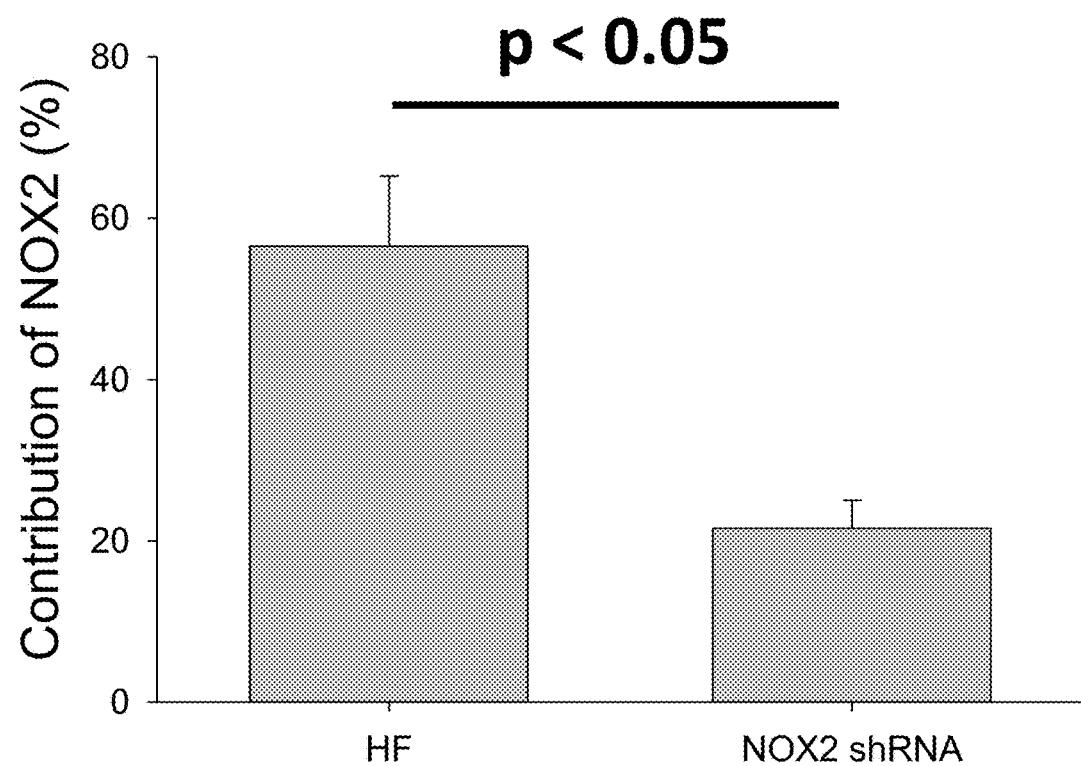


FIG. 10

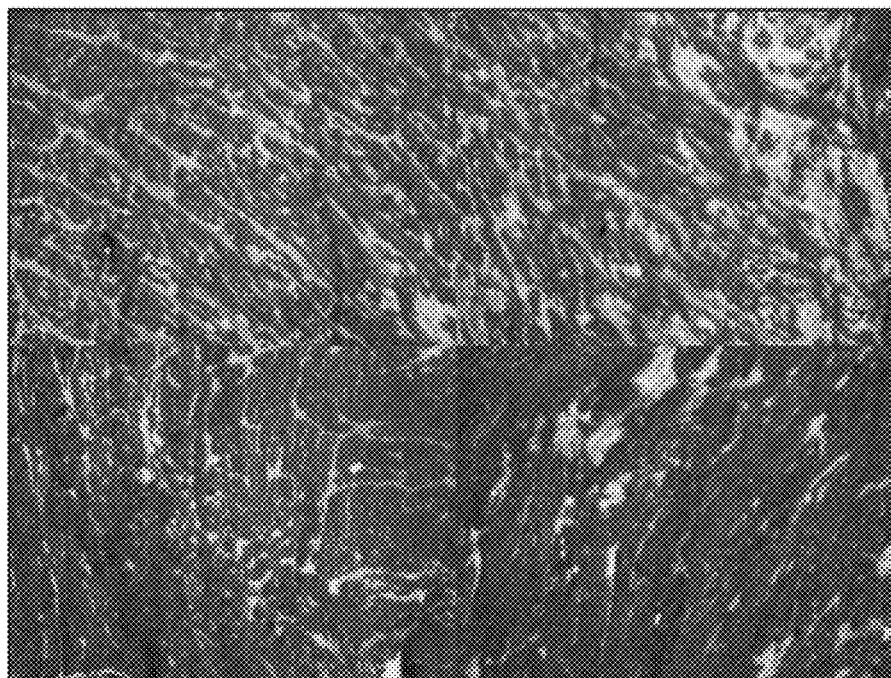


FIG. 11A

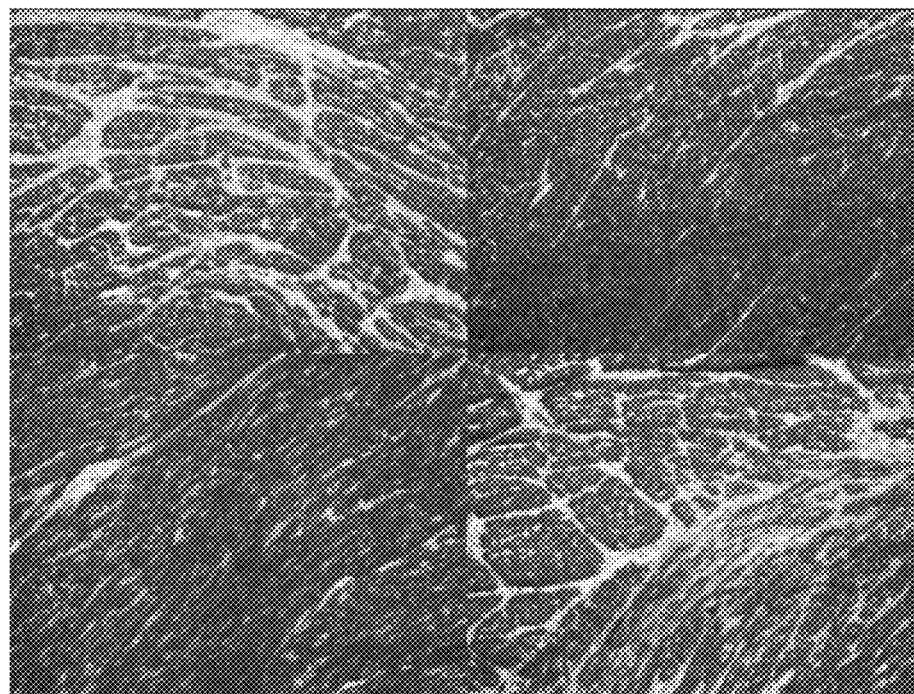


FIG. 11B

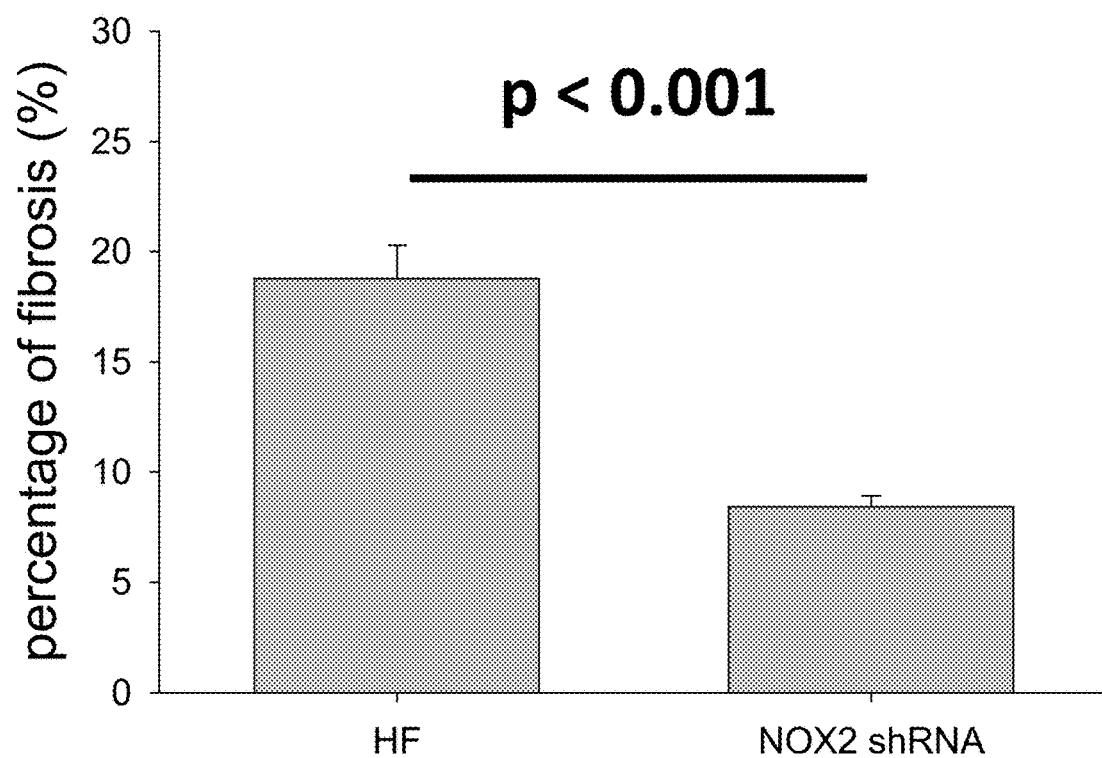


FIG. 11C

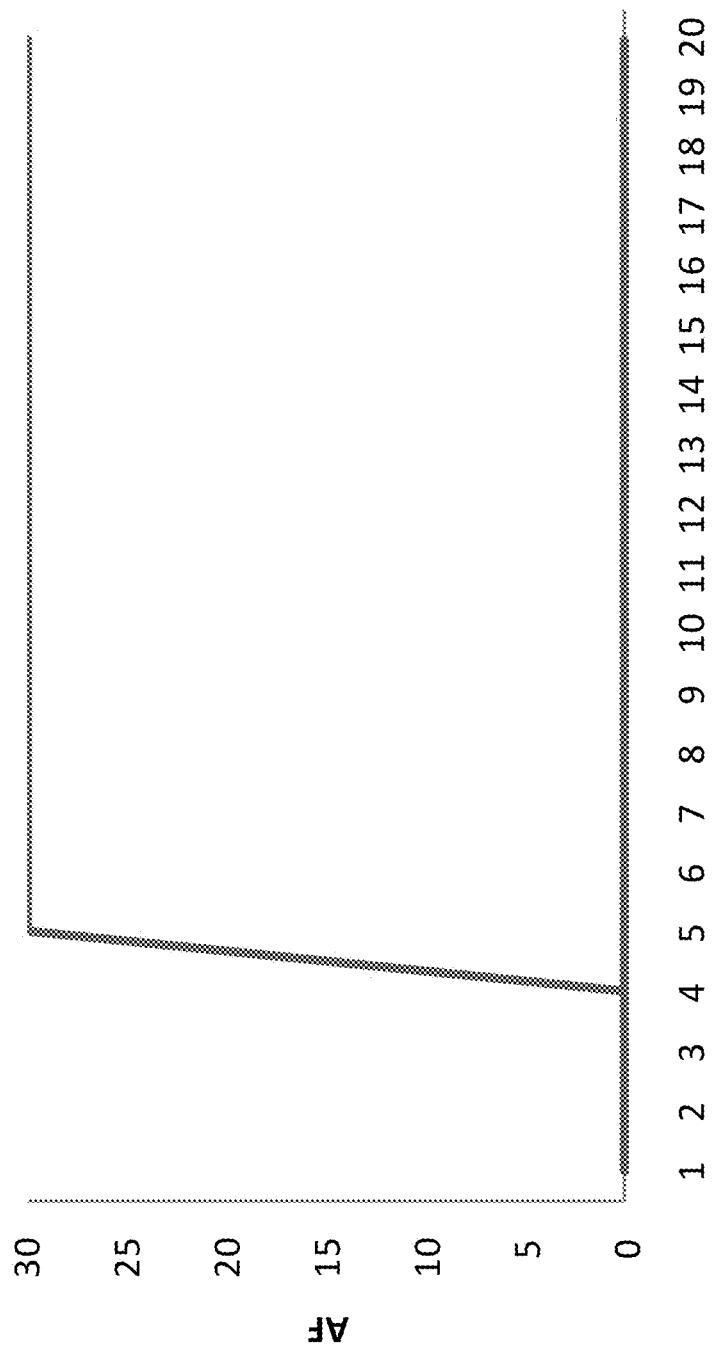


FIG. 12

INHIBITION OF OXIDATIVE STRESS IN ATRIAL FIBRILLATION

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application is a divisional of U.S. non-provisional patent application Ser. No. 14/542,501, now U.S. Pat. No. 9,932,588, issued Apr. 3, 2018, which claims benefit of priority under 35 U.S.C. 119 to U.S. provisional patent application Ser. No. 61/904,925, filed Nov. 15, 2013, and entitled "INHIBITION OF OXIDATIVE STRESS IN ATRIAL FIBRILLATION," the contents of which are herein incorporated by reference in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] This invention was made with government support under 1R01HL093490 awarded by the National Institutes of Health. The government has certain rights in the invention.

FIELD OF INVENTION

[0003] The present disclosure relates to compositions and methods for preventing certain cardiac damage. In particular, compositions and methods of inhibiting oxidative stress in a subject suffering from atrial fibrillation is provided.

BACKGROUND

[0004] Atrial Fibrillation (AF) is the most common heart rhythm disorder (Benjamin E J, Levy D, Vaziri S M, D'Agostino R B, Belanger A J, Wolf P A. "Independent risk factors for atrial fibrillation in a population-based cohort. The Framingham Heart Study," *JAMA* 1994; 271:840-4), and is a major risk factor for stroke and HF (Balasubramanian R, Kistler P M. AF and "Heart failure: the chicken or the egg?" *Heart* 2009; 95:535-9; Lakshminarayan K, Anderson D C, Herzog C A, Qureshi A I. "Clinical epidemiology of atrial fibrillation and related cerebrovascular events in the United States," *Neurologist* 2008; 14:143-50; Lip G Y, Kakar P, Watson T. "Atrial fibrillation—the growing epidemic" [comment], *Heart* 2007; 93:542-3). Since a majority of AF triggers arise in the pulmonary veins (PVs) and the adjoining adjoining posterior left atrium (PLA), ablation procedures that electrically isolate the PVs have emerged in recent years as a viable therapy for focal AF. Nonetheless, moderately high ablation success rates have only been achieved in selected patients (Nademanee K, McKenzie J, Kosar E et al. "A new approach for catheter ablation of atrial fibrillation: mapping of the electrophysiologic substrate" [see comment], *J. Am. Coll. Cardiol.* 2004; 43:2044-53; Nademanee K, Schwab M C, Kosar E M et al. "Clinical outcomes of catheter substrate ablation for high-risk patients with atrial fibrillation," *J. Am. Coll. Cardiol.* 2008; 51:843-9; Taylor G W, Kay G N, Zheng Bishop S, Ideker R E. "Pathological effects of extensive radiofrequency energy applications in the pulmonary veins in dogs," *Circulation* 2000; 101:1736-42). Indeed, in patients with structural heart disease e.g. heart failure (HF), success rates do not exceed 50-60% (Estner H L, Hessling G, Ndreppepa G et al. "Electrogram-guided substrate ablation with or without pulmonary vein isolation in patients with persistent atrial fibrillation," *Europace* 2008; 10:1281-7; Weerasooriya R, Khairy P, Litalien J et al. "Catheter ablation for atrial fibrillation: are results maintained at 5 years of follow-up?" *J. Am. Coll.*

Cardiol. 2011; 57:160-6). One reason for this low efficacy is that current ablation strategies primarily employ an anatomical, 'one-size fits all' strategy (with some minor variations) that does not address the specific mechanisms underlying AF (Ben Morrison T, Jared Bunch T, Gersh B J. "Pathophysiology of concomitant atrial fibrillation and heart failure: implications for management," *Nat. Clin. Pract. Cardiovasc. Med* 2009; 6:46-56). Recent research has therefore attempted to better define the mechanisms underlying AF, in order to improve upon the success of ablation and to develop new biological therapies for AF.

[0005] In the setting of structural heart disease—specifically HF—a variety of mechanisms e.g. stretch, oxidative stress (OS), autonomic imbalance and structural changes such as fibrosis are thought to contribute to a vulnerable AF substrate (Nattel S. "From guidelines to bench: implications of unresolved clinical issues for basic investigations of atrial fibrillation mechanisms," *Can. J. Cardiol.* 2011; 27:19-26; Nattel S, Burstein B, Dobrev D. "Atrial remodeling and atrial fibrillation: mechanisms and implications," *Circ. Arrhythm. Electrophysiol.* 2008; 1:62-73). OS is known to be elevated in the atria in AF (Youn J Y, Zhang J, Zhang Y et al. "Oxidative stress in atrial fibrillation: an emerging role of NADPH oxidase," *J. Mol. Cell. Cardiol.* 2013; 62:72-9) and reactive oxygen species (ROS) have effects on the atrial action potential and Ca^{2+} cycling. However, the precise effects of ROS on atrial electrophysiology in the intact atria—and how these electrophysiological changes contribute to formation of AF substrate—are not known.

[0006] Oxygen derivatives with instabilities and increased reactivity, e.g. O_2 , H_2O_2 , and OH, are generically termed ROS (Maejima Y, Kuroda J, Matsushima S, Ago T, Sadoshima J. "Regulation of myocardial growth and death by NADPH oxidase," *J. Mol. Cell. Cardiol.* 2011; 50:408-16). While ROS at low doses mediates physiological functions such as growth, differentiation, metabolism (id.), excess ROS damages DNA, protein and lipids, and causes cell death (id.). A wealth of research points to increased OS as a key driver of cardiac remodeling caused by chronic pressure overload, loss of functional myocardium or AF (Kohlhaas M, Maack C. "Interplay of defective excitation-contraction coupling, energy starvation, and oxidative stress in heart failure," *Trends Cardiovasc. Med* 2011; 21:69-73; Maulik S K, Kumar S. "Oxidative stress and cardiac hypertrophy: a review," *Toxicol. Mech. Methods* 2012; 22:359-66). In addition, chronic ROS elevation activates signaling pathways such as TGF-1, MAP kinase subfamilies (Hori M, Nishida K. "Oxidative stress and left ventricular remodeling after myocardial infarction," *Cardiovas. Res.* 2009; 81:457-64; Tsai K H, Wang W J, Lin C W et al. "NADPH oxidase-derived superoxide anion-induced apoptosis is mediated via the JNK-dependent activation of NF- κ B in cardiomyocytes exposed to high glucose," *J. Cell. Physiol.* 2012; 227:1347-57) that result in structural changes e.g. fibrosis. Moreover, ROS generation (e.g. by Ang II mediated NOX activation) has been shown to lead to modifications of CaMKII (Erickson J R, He B J, Grumbach I M, Anderson M E. "CaMKII in the cardiovascular system: sensing redox states," *Physiol. Rev.* 2011; 91:889-915), an important serine-threonine kinase involved in a variety of E-C coupling related processes in cardiac myocytes.

[0007] ROS are generated by the mitochondrial electron transport chain, the xanthine oxidase/dehydrogenase system, 'uncoupled' NOS, cytochrome P450 and NADPH oxidases.

The NADPH oxidase enzyme family are a major source of cardiovascular ROS (Murdoch C E, Zhang M, Cave A C, Shah A M. "NADPH oxidase-dependent redox signalling in cardiac hypertrophy, remodelling and failure," *Cardiovasc. Res.* 2006; 71:208-15; Cave A C, Brewer A C, Narayananapicker A et al "NADPH oxidases in cardiovascular health and disease," *Antioxid Redox. Signal* 2006; 8:691-728) with NOX2 being the dominant ROS-generating NADPH isoform in HF (Nabeebaccus A, Zhang M, Shah A M. "NADPH oxidases and cardiac remodeling," *Heart Fail. Rev.* 2011; 16:5-12; Maejima Y, Kuroda J, Matsushima S, Ago T, Sadoshima J. "Regulation of myocardial growth and death by NADPH oxidase," *J Mol. Cell. Cardiol.* 2011; Cave A C, Brewer A C, Narayananapicker A et al. "NADPH oxidases in cardiovascular health and disease," *Antioxid Redox. Signal* 2006; 8:691-728; Zhang P, Hou M, Li Y et al "NADPH oxidase contributes to coronary endothelial dysfunction in the failing heart," *Am. J. Physiol Heart. Circ. Physiol* 2009; 296:H840-6; Dworakowski R, Alom-Ruiz S P, Shah A M. "NADPH oxidase-derived reactive oxygen species in the regulation of endothelial phenotype," *Pharmacol. Rep.* 2008; 60:21-8). However, more recent studies indicate that NOX4 in mitochondria plays an essential role in mediating OS during pressure overload-induced cardiac hypertrophy (Nabeebaccus A, Zhang M, Shah A M. "NADPH oxidases and cardiac remodeling," *Heart Fail. Rev.* 2011; 16:5-12; Kuroda J, Ago T, Matsushima S, Zhai P, Schneider M D, Sadoshima J. "NADPH oxidase 4 (Nox4) is a major source of oxidative stress in the failing heart," *Proc. Natl. Acad Sci. U.S.A.* 2010; 107:15565-70; Zhang M, Brewer A C, Schroder K et al. "NADPH oxidase-4 mediates protection against chronic load-induced stress in mouse hearts by enhancing angiogenesis," *Proc. Natl. Acad Sci. U.S.A.* 2010; 107: 18121-6) NOX4 also appears to contribute significantly to the formation of fibrosis; in addition, it appears to be activated by pro-fibrotic signaling pathways e.g. TGF- β (Yeh Y H, Kuo C T, Chang G J, Qi X Y, Nattel S, Chen W J. "Nicotinamide adenine dinucleotide phosphate oxidase 4 mediates the differential responsiveness of atrial versus ventricular fibroblasts to transforming growth factor-beta," *Circ. Arrhythm. Electrophysiol.* 2013; 6:790-8; Zhang M, Perino A, Ghigo A, Hirsch E, Shah A M. "NADPH oxidases in heart failure: poachers or gamekeepers?" *Antioxid Redox. Signal.* 2013; 18:1024-41).

[0008] Recent evidence indicates that OS also contributes to structural and electrical remodeling in AF. Mihm et al. demonstrated significant oxidative damage in atrial appendages of AF patients undergoing the Maze procedure (Huang C X, Liu Y, Xia W F, Tang Y H, Huang H. Oxidative stress: a possible pathogenesis of atrial fibrillation. *Med Hypotheses* 2009; 72:466-7). Carnes et al showed that dogs with sustained AF had an increase in protein nitration, suggesting enhanced OS (Carnes C A, Janssen P M, Ruehr M L et al. "Atrial glutathione content, calcium current, and contractility," *J. Biol. Chem.* 2007; 282:28063-73; Carnes C A, Chung M K, Nakayama T et al. "Ascorbate attenuates atrial pacing-induced peroxynitrite formation and electrical remodeling and decreases the incidence of postoperative atrial fibrillation," *Circ. Res.* 2001; 89:E32-8). Kim et al showed that NADPH oxidase (NOX2) was a major source of atrial ROS in patients with AF (Kim Y M, Guzik T J, Zhang Y H et al "A myocardial Nox2 containing NAD(P)H oxidase contributes to oxidative stress in human atrial fibrillation," *Circ. Res.* 2005; 97:629-36). More recently, Reilly et al. have

shown that atrial sources of ROS vary with the duration and substrate of AF, with NADPH oxidase being elevated early in AF (e.g. with post-operative AF) and with mitochondrial oxidases and uncoupled NOS being noted in long standing AF (Reilly S N, Jayaram R, Nahar K et al "Atrial sources of reactive oxygen species vary with the duration and substrate of atrial fibrillation: implications for the antiarrhythmic effect of statins," *Circulation* 2011; 124:1107-17). More recent data demonstrates that NOX4—which, as mentioned above, appears to contribute to the generation of mitochondrial ROS, specifically H_2O_2 (which more explicitly promotes fibrosis (Cucoranu I, Clempus R, Dikalova A et al. "NAD(P)H Oxidase 4 Mediates Transforming Growth Factor-1-Induced Differentiation of Cardiac Fibroblasts Into Myofibroblasts," *Circ. Res.* 2005; 97:900-7))—is also elevated in AF (Joun et al. (2013) supra; Zhang J, Youn J Y, Kim A et al. "NOX4-dependent Hydrogen Peroxide Overproduction in Human Atrial Fibrillation and HL-1 Atrial Cells: Relationship to Hypertension," *Front. Physiol.* 2012; 3)

[0009] The detrimental electrical effects of an enhanced, pathological late I_{Na} include the following: (i) diastolic depolarization during phase 4 of the AP that may lead to spontaneous AP firing and abnormal automaticity, (ii) an increase of AP duration, which may lead to EADs and triggered activity, as well as increased spatiotemporal differences of repolarization time, which promote reentrant electrical activity; and (iii) the indirect effects of a late I_{Na} -induced increase of Na^+ entry to alter Ca^{2+} homeostasis in myocytes, which may lead to Ca^{2+} alternans and DADs. Acquired conditions and drugs that enhance late I_{Na} are associated with atrial tachyarrhythmias, ventricular tachyarrhythmias including torsades de pointes (TdP), afterpotentials (EADs, DADs), and triggered activity (Shryock J C, Song Y, Rajamani S, Antzelevitch C, Belardinelli L. "The arrhythmogenic consequences of increasing late I_{Na} in the cardiomyocyte," *Cardiovasc. Res.* 2013; 99:600-11). Recent investigations implicate a role for abnormal Ca^{2+} handling in the genesis of ventricular and atrial arrhythmias (Astrup G L, Balke C W, Wasserstrom J A. Arrhythmia triggers in heart failure: the smoking gun of $[Ca^{2+}]_i$ dysregulation," *Heart Rhythm.* 2011; 8:1804-8; Antoons G, Sipido K R. "Targeting calcium handling in arrhythmias," *Europace* 2008; 10:1364-9; Laurita K R, Rosenbaum D S. "Mechanisms and potential therapeutic targets for ventricular arrhythmias associated with impaired cardiac calcium cycling," *J. Mol. Cell. Cardiol.* 2008; 44:31-43). Abnormal Ca^{2+} handling can contribute to arrhythmogenesis directly by triggering abnormal depolarizations and indirectly by modulating action potential time course and duration. DADs are typically result from cellular Ca^{2+} overload, with SCR increasing forward NCX and producing an inward current resulting in DADs (Antoons et al. (2008) supra; Volders P G, Vos M A, Szabo B et al "Progress in the understanding of cardiac early afterdepolarizations and torsades de pointes: time to revise current concepts," *Cardiovasc. Res.* 2000; 46:376-92). More recent evidence has accumulated for Ca^{2+} -mediated EADs (Volders et al. (2000) supra), which could contribute to triggered activity or at least prolong action potential duration. Indeed, abnormal Ca^{2+} cycling and resulting Ca^{2+} transient alternans predisposes to changes in the action potential that set up conditions for reentry (Laurita et al. (2008) supra). Abnormal SR Ca^{2+} release has also been suggested to contribute to reentry in the intact atria, includ-

ing the PVs (Chou C C, Nihei M, Zhou S et al. "Intracellular calcium dynamics and anisotropic reentry in isolated canine pulmonary veins and left atrium," *Circulation* 2005; 111: 2889-97). In HF, a number of atrial ion-channel and E-C coupling proteins (Li D, Melnyk P, Feng J et al. "Effects of Experimental Heart Failure on Atrial Cellular and Ionic Electrophysiology," *Circulation* 2000; 101:2631-8. 61. Yeh Y-H, Wakili R, Qi X-Y et al. "Calcium-Handling Abnormalities Underlying Atrial Arrhythmogenesis and Contractile Dysfunction in Dogs With Congestive Heart Failure," *Circ. Arrhythm. Electrophysiol.* 2008; 1:93-102) can be modulated by ROS (Hool L C. "Reactive Oxygen Species in Cardiac Signalling: From Mitochondria to Plasma Membrane Ion Channels," *Clin. Exp. Pharm. Phys.* 2006; 33:146-51; Zima A V, Blatter L A. "Redox regulation of cardiac calcium channels and transporters," *Cardiovasc. Res.* 2006; 71:310-21; Nediani C, Raimondi L, Borchi E, Cerbai E. "Nitric Oxide/Reactive Oxygen Species Generation and Nitroso/Redox Imbalance in Heart Failure: From Molecular Mechanisms to Therapeutic Implications," *Antioxidants & Redox Signaling* 2011; 14:289-331) at least in part via ROS activation of kinases and inactivation of phosphatases, resulting in aberrant phosphorylation (e.g. of RyR2 and phospholamban). Also, ROS directly decrease SERCA function, but increase NCX function (Kuster G M, Lancel S, Zhang J et al. "Redox-mediated reciprocal regulation of SERCA and Na⁺-Ca²⁺ exchanger contributes to sarcoplasmic reticulum Ca²⁺ depletion in cardiac myocytes," *Free Rad Biol. Med.* 2010; 48:1182-7) which parallels the changes in SERCA and NCX in HF. Additionally, ROS increases late/persistent I_{Na} (I_{Na} late) (Luo A, Ma J, Zhang P, Zhou H, Wang W. "Sodium Channel Gating Modes During Redox Reaction," *Cell. Phys. Bioch.* 2007; 19:9-20), which again parallels that in HF (Valdivia C R, Chu W W, Pu J et al. "Increased late sodium current in myocytes from a canine heart failure model and from failing human heart," *J. Mol. Cell. Cardiol.* 2005; 38:475-83) and I_{Na}, late can significantly contribute to the induction of EADs and DADs (Li D, Melnyk P, Feng J et al. "Effects of Experimental Heart Failure on Atrial Cellular and Ionic Electrophysiology," *Circulation* 2000; 101:2631-8; Song Y, Shryock J C, Belardinelli L. "An increase of late sodium current induces delayed afterdepolarizations and sustained triggered activity in atrial myocytes," *Am. J. Physiol-Heart and Circ. Physiol.* 2008; 294:H2031-H9; Wasserstrom J A, Sharma R, O'Toole M J et al. "Ranolazine Antagonizes the Effects of Increased Late Sodium Current on Intracellular Calcium Cycling in Rat Isolated Intact Heart," *J. Pharm. Exp. Ther.* 2009; Undrovinas N, Maltsev V, Belardinelli L, Sabbah H, Undrovinas A. "Late sodium current contributes to diastolic cell Ca²⁺ accumulation in chronic heart failure," *J. Physiol. Sci.* 2010; 60:245-57) both in ventricles and atria. Additional promotion of triggered activity could come from the increased Ca²⁺ sensitivity of hyperphosphorylated RyR2s in HF (Terentyev D, Gyorko I, Belevych A E et al. "Redox Modification of Ryanodine Receptors Contributes to Sarcoplasmic Reticulum Ca²⁺ Leak in Chronic Heart Failure," *Circ. Res.* 2008; 103:1466-72), which together with OS modifications of RyR2(nitrosylation, oxidation) (Valdivia C R, Chu W W, Pu J et al "Increased late sodium current in myocytes from a canine heart failure model and from failing human heart," *J. Mol. Cell. Card.* 2005; 38:475-83. 65. Song Y, Shryock J C, Belardinelli L. "An increase of late sodium current induces

delayed after depolarizations and sustained triggered activity in atrial myocytes," *Am. J. Physiol. Heart Circ. Physiol.* 2008; 294:H2031-H9) in HF lead to leaky ventricular RyR2s (Gonzalez D R, Beigi F, Treuer A V, Hare J M. "Deficient ryanodine receptor S-nitrosylation increases sarcoplasmic reticulum calcium leak and arrhythmogenesis in cardiomyocytes," *Proc. Natl. Acad. Sci. U.S.A.* 2007; 104:20612-7; Marx S O, Marks A R. "Dysfunctional ryanodine receptors in the heart: new insights into complex cardiovascular diseases," *J. Mol. Cell. Cardiol.* 2013; 58:225-31). The oxidation/nitrosylation state of atrial RyR2s in HF has not been fully scrutinized; however, there appear to be significant differences in atrial versus ventricular E-C coupling, as has previously been suggested by others (Bootman M D, Smyrnias I, Thul R, Coombes S, Roderick H L. "Atrial cardiomyocyte calcium signaling," *Biochim. Biophys. Acta* 2011; 1813:922-34). The majority of the studies mentioned above have been performed in isolated myocytes; the specific contribution oxidized I_{Na}, RyR₂ to the electrophysiological characteristics of the intact atrium—and how this contributes to arrhythmogenesis—is not known.

[0010] Excessively activated CaMKII is implicated in the genesis of HF and arrhythmias. Recent evidence suggests that both CaMKII and H₂O₂ increase RyR2 (Marx S O, Marks A R. "Dysfunctional ryanodine receptors in the heart: new insights into complex cardiovascular diseases," *J. Mol. Cell. Cardiol.* 2013; 58:225-31; Niggli E, Ullrich N D, Gutierrez D, Kyrychenko S, Polakova E, Shirokova N. "Posttranslational modifications of cardiac ryanodine receptors: Ca(2+) signaling and EC-coupling," *Biochim. Biophys. Acta* 2013; 1833:866-75) P_O (Undrovinas N, Maltsev V, Belardinelli L, Sabbah H, Undrovinas A. "Late sodium current contributes to diastolic cell Ca²⁺ accumulation in chronic heart failure," *J. Physiol. Sci.* 2010; 60:245-57; Gonzalez D R, Beigi F, Treuer A V, Hare J M. "Deficient ryanodine receptor S-nitrosylation increases sarcoplasmic reticulum calcium leak and arrhythmogenesis in cardiomyocytes," *Proc. Natl. Acad. Sci. U.S.A.* 2007; 104:20612-7; Donoso P, Sanchez G, Bull R, Hidalgo C. "Modulation of cardiac ryanodine receptor activity by ROS and RNS," *Front. Biosci. (Landmark Ed)* 2011; 16:553-67; Terentyev D, Gyorko I, Belevych A E, et al. "Redox modification of ryanodine receptors contributes to sarcoplasmic reticulum Ca²⁺ leak in chronic heart failure," *Circ. Res.* 2008; 103:1466-72), thereby promoting SR Ca²⁺ leak and arrhythmias (Belevych A E, Terentyev D, Terentyeva R et al. "Shortened Ca²⁺ signaling refractoriness underlies cellular arrhythmogenesis in a postinfarction model of sudden cardiac death," *Circ. Res.* 2012; 110:569-77). Since OS-mediated oxidation of Met 281/282 residues in the regulatory domain of CaMKII transforms CaMKII into a constitutively active form (ox-CaMKII), leading to aberrant phosphorylation of multiple E-C coupling proteins (Swaminathan P D, Purohit A, Hund T J, Anderson M E. "Calmodulin-dependent protein kinase II: linking heart failure and arrhythmias," *Circ. Res.* 2012; 110:1661-77; Erickson J R, He B J, Grumbach I M, Anderson M E. "CaMKII in the cardiovascular system: sensing redox states," *Physiol. Rev.* 2011; 91:889-915), OS may affect RyR₂P_O both directly (via ROS) and indirectly (via ox-CaMKII) Indeed, expression of ox-CaMKII has been found to be increased in atria of AF patients, indicating a potential role of ROS induced CaMKII activation in AF (Purohit A, Rokita A G, Guan X et al. "Oxidized Ca2+/Calmodulin-Dependent Pro-

tein Kinase II Triggers Atrial Fibrillation," *Circulation* 2013; 128:1748-57). CaMKII has also been shown to modulate the gating of $Na_v1.5$, at least in part by phosphorylation of $Na_v1.5$ at multiple sites (Ashpole N M, Herren A W, Ginsburg K S et al. "Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) regulates cardiac sodium channel NaV1.5 gating by multiple phosphorylation sites," *J. Biol. Chem.* 2012; 287:19856-69). A recent study demonstrates a fundamental requirement for targeting of CaMKII to a controlling phosphorylation site, S571, on $Na_v1.5$ (Hund T J, Koval O M, Li J et al. "A beta(IV)-spectrin/CaMKII signaling complex is essential for membrane excitability in mice," *J. Clin. Invest.* 2010; 120:3508-19).

[0011] CaMKII phosphorylation of $Na_v1.5$ is thought to decrease transient I_{Na} , but increase $I_{Na,late}$, again thereby contributing to the genesis of triggered activity (Hashambhoy Y L, Winslow R L, Greenstein J L. "CaMKII-dependent activation of late INa contributes to cellular arrhythmia in a model of the cardiac myocyte," *Conf Proc. IEEE Eng Med Biol. Soc.* 2011; 2011:4665-8). Modeling studies also suggest that ox-CaMKII may create substrate for reentry by regulating conduction characteristics of the myocardium (Hashambhoy et al. (2011) *supra*; Christensen M D, Dun W, Boyden P A, Anderson M E, Mohler P J, Hund T J. "Oxidized calmodulin kinase II regulates conduction following myocardial infarction: a computational analysis," *PLoS Comput. Biol.* 2009; 5:e1000583), with this substrate thought to be at least partially mediated by modulation of I_{Na} .

[0012] A summary of the various mechanisms for reactive oxygen species production, oxidative stress generation, and development of fibrosis and atrial fibrillation is illustrated in FIG. 1.

SUMMARY

[0013] In a first respect, a method of inhibiting oxidative stress in a subject having atrial or ventricular arrhythmias, ventricular failure or heart failure is provided. The method includes the step of administering an effective amount of a NOX2 inhibitor agent to the subject, wherein said administering is under conditions such that a level of oxidative stress in myocardial tissue is reduced or eliminated.

[0014] In a second respect, a method of treating a subject having atrial or ventricular arrhythmias, ventricular failure or heart failure is provided. The method includes the step of administering an effective amount of a NOX2 inhibitor agent to the subject, wherein said administering is under conditions such that a level of atrial or ventricular arrhythmias is reduced or eliminated.

[0015] In a third respect, a pharmaceutical composition for inhibiting oxidative stress in a subject having atrial or ventricular arrhythmias, ventricular failure or heart failure is provided. The pharmaceutical composition includes an isolated nucleic acid encoding a small hairpin RNA against NOX2 mRNA (NOX2 rhRNA).

[0016] These and other features, objects and advantages of the present invention will become better understood from the description that follows. In the description, reference is made to the accompanying drawings, which form a part hereof and in which there is shown by way of illustration, not limitation, embodiments of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0017] FIG. 1 depicts a scheme showing various mechanisms for reactive oxygen species production, oxidative stress generation, and development of fibrosis and atrial fibrillation.

[0018] FIG. 2A depicts O_2^- production, as measured by lucigenin enhanced chemiluminescence assay, for left atrial appendage (LAA; light blue bars) and posterior left atrium (PLA; dark blue bars) for normal and HF animals. Statistics key: *, p<0.05; **, p<0.001.

[0019] FIG. 2B depicts O_2^- production (as measured by lucigenin enhanced chemiluminescence assay) in PLA for NADPH oxidase (blue bars), Mitochondrial ROS (red bars), NOS (green bars) and Xanthine oxidase (purple bars) from control and HF animals.

[0020] FIG. 3A shows, using western blotting with an anti-gp91 antibody that NOX2 (91-kDa protein subunit) was increased in the HF PLA relative control PLA (GAPDH control protein (38-kDa) is shown for comparison to normalize for loading differences in the gel lanes).

[0021] FIG. 3B depicts quantitative results of anti-gp91 antibody (NOX2) data of FIG. 3A.

[0022] FIG. 4 shows an exemplary expression vector for expression a NOX2 shRNA under the control of a U6 PolIII promoter.

[0023] FIG. 5 shows that the duration of AF (seconds) is decreased in plasmid encoding NOX2 shRNA-treated animals ("NOX2 shRNA") compared with lacZ-transfected control animals ("HF (LacZ Control)").

[0024] FIG. 6a shows increased expression of ox-CAMKII in PLA of control HF dogs (green fluorescence represents ox-CAMKII).

[0025] FIG. 6B shows decreased expression of ox-CAMKII in the PLA of HF dogs transfected with NOX2 shRNA (green fluorescence represents ox-CAMKII).

[0026] FIG. 7 depicts changes in ERP in plasmid encoding NOX2 shRNA-treated animals (NOX2 shRNA) compared with lacZ-transfected control animals (HF) for baseline (black bars) and post-pacing (grey bars) conditioned animals.

[0027] FIG. 8A depicts conduction inhomogeneity index (3.25) for PLA from control HF animals.

[0028] FIG. 8B depicts conduction inhomogeneity index (1.5) for PLA from animals transfected with plasmid encoding NOX2 rhRNA in PLA tissue.

[0029] FIG. 8C depicts statistical differences in conduction inhomogeneity indices for PLA from HF animals at day 1 (black bars) and termination (grey bars).

[0030] FIG. 8D depicts no statistical difference in conduction inhomogeneity indices for PLA from animals transfected with plasmid encoding NOX2 rhRNA in PLA tissue.

[0031] FIG. 9A depicts rtPCR analysis of NOX2 mRNA in PLA from control HF animals as compared to animals with plasmid encoding NOX2 shRNA-transfected PLA.

[0032] FIG. 9B depicts representative NOX2 protein levels in PLA from control HF animals as compared to animals with plasmid encoding NOX2 shRNA-transfected PLA (cadherin used as a control protein).

[0033] FIG. 10 depicts representative O_2^- levels attributed to NADPH oxidase (NOX2) in PLA from control HF animals as compared to animals with plasmid encoding NOX2 shRNA-transfected PLA.

[0034] FIG. 11A depicts the extent of fibrosis in PLA from control HF animals.

[0035] FIG. 11B depicts the extent of fibrosis in PLA from animals with plasmid encoding NOX2 shRNA-transfected PLA.

[0036] FIG. 11C depicts a quantitative analysis of the percentage of fibrosis in PLA from control HF animals transfected with control plasmid (that is, not encoding NOX2 shRNA).

[0037] FIG. 12 depicts the onset of AF in a rapid atrial pacing canine model where three control animals experienced sustained AF (blue line) compared with the absence of AF onset in two animals with plasmid encoding NOX2 shRNA-transfected PLA (red line).

DETAILED DESCRIPTION

[0038] The present disclosure provides details of the discovery of pharmaceutical compositions and methods for inhibiting oxidative stress in a subject suffering from atrial fibrillation. The method involves targeting reducing reactive oxygen species (ROS) generated through inhibiting the action of NADPH oxidase. The principle is demonstrated using an NOX2 inhibitor based upon RNA interference (RNAi) with small hairpin RNAs directed against the NOX2 mRNA (NOX2 shRNA). Pharmaceutical compositions based upon NOX2 shRNA is shown in the method to inhibit expression of the NOX2 gene, thereby leading to decreased production of NADPH oxidase in cardiac cells containing the injected NOX2 shRNAs. Referring to FIG. 1, lower NADPH oxidase production can lead to lower levels of ROS produced, lower extents of oxidative stress, decreased extents of atrial fibrosis and lower atrial fibrillation (AF). However, one skilled in the art will appreciate that the principle can be readily extended to other NOX2 inhibitors with undue experimentation and have a reasonable expectation of achieving similar results as described herein. Details of the pharmaceutical compositions and methods are presented in greater detail in this disclosure.

Terminology and Definitions

[0039] The terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting. With respect to the use of substantially, any plural and/or singular terms herein, those having skill in the art can translate from the plural as is appropriate to the context and/or application. The various singular/plural permutations may be expressly set forth herein for the sake of clarity.

[0040] Terms used herein are intended as "open" terms (e.g., the term "including" should be interpreted as "including but not limited to," the term "having" should be interpreted as "having at least," the term "includes" should be interpreted as "includes but is not limited to," etc.).

[0041] The phrase "such as" should be interpreted as "for example, including."

[0042] Furthermore, in those instances where a convention analogous to "at least one of A, B and C, etc." is used, in general such a construction is intended in the sense of one having ordinary skill in the art would understand the convention (e.g., "a system having at least one of A, B and C" would include but not be limited to systems that have A alone, B alone, C alone, A and B together, A and C together, B and C together, and/or A, B, and C together.). It will be further understood by those within the art that virtually any disjunctive word and/or phrase presenting two or more

alternative terms, whether in the description or figures, should be understood to contemplate the possibilities of including one of the terms, either of the terms, or both terms. For example, the phrase "A or B" will be understood to include the possibilities of "A" or 'B or "A and B."

[0043] All language such as "up to," "at least," "greater than," "less than," and the like, include the number recited and refer to ranges which can subsequently be broken down into subranges as discussed above.

[0044] A range includes each individual member. Thus, for example, a group having 1-3 members refers to groups having 1, 2, or 3 members. Similarly, a group having 6 members refers to groups having 1, 2, 3, 4, or 6 members, and so forth.

[0045] The phrase "small hairpin RNA" and the term "shRNA", as used herein, refer to a unimolecular RNA-containing polynucleotide that is capable of performing RNAi and that includes a sense sequence, a loop, and an antisense sequence. The sense and antisense sequences are sometimes referred to herein as the first region and second region. As described herein, the sense and antisense sequences can be in different orientations with respect to one another in an shRNA of the invention (an L or R shRNA). Thus, if the first region of an shRNA is the sense sequence then the second region is the antisense region, and vice versa. Preferably, the sense and antisense sequences are substantially complementary to each other (about 80% complementary). The antisense sequence can be about 16 to about 22 nucleotides in length, e.g., about 16 to 19 nucleotides, and more preferably 18 to 19 nucleotides in length. The sense sequence can be about 11 to about 22 nucleotides in length, and more preferably 17 to 19 nucleotides in length. An shRNA (and other RNAi agents) are "specific" for a target gene when the antisense sequence (of about 16 to 22 nucleotides) is substantially complementary to the target gene (or target RNA, e.g., target mRNA). By substantially complementary is meant that the antisense sequence is at least 80% complementary to the target gene (or gene product). Thus, in some embodiments, the antisense sequence that is complementary to the target gene can contain mismatches to the target. The sequence can be varied to target one or more genetic variants or phenotypes of a target, e.g., a viral target, by altering the targeting sequence to be complementary to the sequence of the genetic variant or phenotype. An shRNA may have a loop as long as, for example, 0 to about 24 nucleotides in length, preferably 0 to about 10 nucleotides in length, 0 to 6 nucleotides in length, e.g., 2 nucleotides in length. The sequence of the loop can include nucleotide residues unrelated to the target. In one particularly preferred embodiment, the loop is 5'-UU-3'. In some embodiments it may include non-nucleotide moieties. In yet other embodiments, the loop does not include any non-nucleotides moieties. Optionally, the shRNA can have an overhang region of 2 bases on 3' end of the molecule. The shRNA can also comprise RNAs with stem-loop structures that contain mismatches and/or bulges. The sense sequence that is homologous to the target can differ at about 0 to about 5 sites by having mismatches, insertions, or deletions of from about 1 to about 5 nucleotides, as is the case, for example, with naturally occurring microRNAs. RNAs that comprise any of the above structures can include structures where the loops comprise nucleotides, non-nucleotides, or combinations of nucleotides and non-nucleotides.

[0046] Additionally, the phrase “small hairpin RNA” and the term “shRNA” include nucleic acids that also contain moieties other than ribonucleotide moieties, including, but not limited to, modified nucleotides, modified internucleotide linkages, non-nucleotides, deoxynucleotides and analogs of the nucleotides mentioned thereof.

[0047] Additionally, the term “L shRNA” refers to an shRNA comprising a sense sequence that is connected through a loop to the 3' end of the antisense.

[0048] Additionally, the term “R shRNA” refers to an shRNA molecule comprising an antisense sequence that is connected through a loop to the 3' end of the sense sequence.

[0049] The phrase “antisense sequence”, as used herein, refers to a polynucleotide or region of a polynucleotide that is substantially complementary (e.g., 80% or more) or 100% complementary to a target nucleic acid of interest. An antisense sequence can be composed of a polynucleotide region that is RNA, DNA or chimeric RNA/DNA. Any nucleotide within an antisense sequence can be modified by including substituents coupled thereto, such as in a 2' modification. The antisense sequence can also be modified with a diverse group of small molecules and/or conjugates. For example, an antisense sequence may be complementary, in whole or in part, to a molecule of messenger RNA, an RNA sequence that is not mRNA (e.g., tRNA, rRNA, hnRNA, negative and positive stranded viral RNA and its complementary RNA) or a sequence of DNA that is either coding or non-coding.

[0050] The phrase “sense sequence”, as used herein, refers to a polynucleotide or region that has the same nucleotide sequence, in whole or in part, as a target nucleic acid such as a messenger RNA or a sequence of DNA. When a sequence is provided, by convention, unless otherwise indicated, it is the sense sequence (or region), and the presence of the complementary antisense sequence (or region) is implicit.

[0051] The term “complementary”, as used herein, refers to the ability of polynucleotides to form base pairs with one another. Base pairs are typically formed by hydrogen bonds between nucleotide units in antiparallel polynucleotide strands or regions. Complementary polynucleotide strands or regions can base pair in the Watson-Crick manner (e.g., A to T, A to U, C to G), or in any other manner that allows for the formation of stable duplexes.

[0052] “Perfect complementarity” or “100% complementarity”, as used herein, refers to the situation in which each nucleotide unit of one polynucleotide strand or region can hydrogen bond with each nucleotide unit of a second polynucleotide strand or region. Less than perfect complementarity refers to the situation in which some, but not all, nucleotide units of two strands or two regions can hydrogen bond with each other. For example, for two 19-mers, if 17 base pairs on each strand or each region can hydrogen bond with each other, the polynucleotide strands exhibit 89.5% complementarity. Substantial complementarity refers to polynucleotide strands or regions exhibiting about 80% or greater complementarity.

[0053] The term “deoxynucleotide”, as used herein, refers to a nucleotide or polynucleotide lacking an OH group at the 2' or 3' position of a sugar moiety with appropriate bonding and/or 2', 3' terminal dideoxy, instead having a hydrogen bonded to the 2' and/or 3' carbon.

[0054] The terms “deoxyribonucleotide” and “DNA”, as used herein, refer to a nucleotide or polynucleotide com-

prising at least one ribosyl moiety that has an H at its 2' position of a ribosyl moiety instead of an OH.

[0055] In some embodiments, an shRNA described herein optionally includes at least one conjugate moiety.

[0056] The term “alkyl”, as used herein, refers to a hydrocarbyl moiety that can be saturated or unsaturated, and substituted or unsubstituted. It may comprise moieties that are linear, branched, cyclic and/or heterocyclic, and contain functional groups such as ethers, ketones, aldehydes, carboxylates, etc. Unless otherwise specified, alkyl groups are not cyclic, heterocyclic, or comprise functional groups.

[0057] Exemplary alkyl groups include, but are not limited to, substituted and unsubstituted groups of methyl, ethyl, propyl, butyl, pentyl, hexyl, heptyl, octyl, nonyl, decyl, undecyl, dodecyl, tridecyl, tetradecyl, pentadecyl, hexadecyl, heptadecyl, octadecyl, nonadecyl, eicosyl and alkyl groups of higher number of carbons, as well as 2-methylpropyl, 2-methyl-4-ethylbutyl, 2,4-diethylpropyl, 3-propylbutyl, 2,8-dibutyldecyl, 6,6-dimethyloctyl, 6-propyl-6-butylloctyl, 2-methylbutyl, 2-methylpentyl, 3-methylpentyl, and 2-ethylhexyl. The term alkyl also encompasses alkenyl groups, such as vinyl, allyl, aralkyl and alkynyl groups. Unless otherwise specified, alkyl groups are not substituted.

[0058] Substitutions within an alkyl group, when specified as present, can include any atom or group that can be tolerated in the alkyl moiety, including but not limited to halogens, sulfurs, thiols, thioethers, thioesters, amines (primary, secondary, or tertiary), amides, ethers, esters, alcohols and oxygen. The alkyl groups can by way of example also comprise modifications such as azo groups, keto groups, aldehyde groups, carboxyl groups, nitro, nitroso or nitrile groups, heterocycles such as imidazole, hydrazine or hydroxylamino groups, isocyanate or cyanate groups, and sulfur containing groups such as sulfoxide, sulfone, sulfide, and disulfide. Unless otherwise specified, alkyl groups do not comprise halogens, sulfurs, thiols, thioethers, thioesters, amines, amides, ethers, esters, alcohols, oxygen, or the modifications listed above.

[0059] Further, alkyl groups may also contain hetero substitutions, which are substitutions of carbon atoms, by for example, nitrogen, oxygen or sulfur. Heterocyclic substitutions refer to alkyl rings having one or more heteroatoms. Examples of heterocyclic moieties include but are not limited to morpholino, imidazole, and pyrrolidino. Unless otherwise specified, alkyl groups do not contain hetero substitutions or alkyl rings with one or more heteroatoms (i.e., heterocyclic substitutions).

[0060] The preferred alkyl group for a 2' modification is a methyl group with an O-linkage to the 2' carbon of a ribosyl moiety, i.e., a 2'-O-alkyl that comprises a 2'-O-methyl group.

[0061] The phrase “2'-O-alkyl modified nucleotide”, as used herein, refers to a nucleotide unit having a sugar moiety, for example a deoxyribosyl moiety that is modified at the 2' position such that an oxygen atom is attached both to the carbon atom located at the 2' position of the sugar and to an alkyl group. In various embodiments, the alkyl moiety consists essentially of carbons and hydrogens. A particularly preferred embodiment is one wherein the alkyl moiety is methyl.

[0062] As used herein, the term “2' carbon modification” refers to a nucleotide unit having a sugar moiety, for example a moiety that is modified at the 2' position of the sugar subunit. A “2'-O-alkyl modified nucleotide” is modified at this position such that an oxygen atom is attached

both to the carbon atom located at the 2' position of the sugar and to an alkyl group, e.g., 2'-O-methyl, 2'-O-ethyl, 2'-O-propyl, 2'-O-isopropyl, 2'-O-butyl, 2'-O-isobutyl, 2'-O-ethyl-O-methyl(-OCH₂CH₂OCH₃), and 2'-O-ethyl-OH(-OCH₂CH₂OH). A "2' carbon sense sequence modification", as used herein, refers to a modification at the 2' carbon position of a nucleotide on the sense sequence. A "2' carbon antisense sequence modification", as used herein, refers to a modification at the 2' carbon position of a nucleotide on the antisense sequence.

[0063] The term "nucleotide", as used herein, refers to a ribonucleotide or a deoxyribonucleotide or modified from thereof, as well as an analog thereof. Nucleotides include species that comprise purines, e.g., adenine, hypoxanthine, guanine, and their derivatives and analogs, as well as pyrimidines, e.g., cytosine, uracil, thymine, and their derivatives and analogs. Preferably, a "nucleotide" comprises a cytosine, uracil, thymine, adenine, or guanine moiety.

[0064] Nucleotide analogs include nucleotides having modifications in the chemical structure of the base, sugar and/or phosphate, including, but not limited to, 5-position pyrimidine modifications, 8-position purine modifications, modifications at cytosine exocyclic amines, and substitution of 5-bromo-uracil; and 2'-position sugar modifications, including but not limited to, sugar-modified ribonucleotides in which the 2'-OH is replaced by a group such as an H, OR, R, halo, SH, SR, NH.sub.2, NHR, NR.sub.2, or CN, wherein R is an alkyl moiety as defined herein. Nucleotide analogs also include nucleotides with bases such as inosine, queuosine, xanthine, sugars such as 2'-methyl ribose, non-natural phosphodiester linkages such as methylphosphonates, phosphorothioates and peptides.

[0065] Modified bases refer to nucleotide bases such as, for example, adenine, guanine, cytosine, thymine, and uracil, xanthine, inosine, and queuosine that have been modified by the replacement or addition of one or more atoms or groups. Some examples of types of modifications include nucleotides that are modified with respect to the base moieties, include but are not limited to, alkylated, halogenated, thiolated, aminated, amidated, or acetylated bases, in various combinations. More specific modified bases include, for example, 5-propynyluridine, 5-propynylcytidine, 6-methyladenine, 6-methylguanine, N,N,-dimethyladenine, 2-propyladenine, 2-propylguanine, 2'-aminoadenine, 1-methylinosine, 3-methyluridine, 5-methylcytidine, 5-methyluridine and other nucleotides having a modification at the 5 position, 5-(2-amino)propyluridine, 5-haloxytidine, 5-halouridine, 4-acetylcytidine, 1-methyladenosine, 2-methyladenosine, 3-methylcytidine, 6-methyluridine, 2-methylguanosine, 7-methylguanosine, 2,2-dimethylguanosine, 5-methylaminoethyluridine, 5-methoxyuridine, deazanucleotides such as 7-deaza-adenosine, 6-azouridine, 6-azocytidine, 6-azothymidine, 5-methyl-2-thiouridine, other thio bases such as 2-thiouridine and 4-thiouridine and 2-thiocytidine, dihydrouridine, pseudouridine, queuosine, archaeosine, naphthyl and substituted naphthyl groups, any O- and N-alkylated purines and pyrimidines such as N6-methyladenosine, 5-methylcarbonylmethyluridine, uridine 5-oxy-acetic acid, pyridine-4-one, pyridine-2-one, phenyl and modified phenyl groups such as aminophenol or 2,4,6-trimethoxy benzene, modified cytosines that act as G-clamp nucleotides, 8-substituted adenines and guanines, 5-substituted uracils and thymines, azapyrimidines, carboxyhydroxalkyl nucleotides, carboxyalkylaminoalkyl nucleo-

tides, and alkylcarbonylalkylated nucleotides. Modified nucleotides also include those nucleotides that are modified with respect to the sugar moiety, as well as nucleotides having sugars or analogs thereof that are not ribosyl. For example, the sugar moieties may be, or be based on, mannoses, arabinoses, glucopyranoses, galactopyranoses, 4-thiouridine, and other sugars, heterocycles, or carbocycles. The term nucleotide analog also includes what are known in the art as universal bases. By way of example, universal bases include but are not limited to 3-nitropyrrole, 5-nitroindole, or nebularine.

[0066] Further, the term nucleotide analog also includes those species that have a detectable label, such as, for example, a radioactive or fluorescent moiety, or mass label attached to the nucleotide.

[0067] The term "overhang", as used herein, refers to terminal non-base pairing nucleotide(s) resulting from one strand or region extending beyond the terminus of the complementary strand to which the first strand or region forms a duplex. One or more polynucleotides that are capable of forming a duplex through hydrogen bonding can have overhangs. The single-stranded region extending beyond the 3' end of the duplex is referred to as an overhang.

[0068] The term "ribonucleotide" and the phrase "ribonucleic acid" (RNA), as used herein, refer to a modified or unmodified nucleotide or polynucleotide comprising at least one ribonucleotide unit. A ribonucleotide unit comprises an oxygen attached to the 2' position of a ribosyl moiety having a nitrogenous base attached in N-glycosidic linkage at the 1' position of a ribosyl moiety, and a moiety that either allows for linkage to another nucleotide or precludes linkage.

[0069] The phrase "heating and snap cooling", as used herein, refers to a two-step procedure that involves heat-denaturing nucleic acids in a sample followed by rapid cooling. For example, tubes that contain shRNA solutions are denatured in a 95.degree. C. heat block for 4 to 5 minutes followed by immediately placing the tubes into an ice-water bath for 30 minutes. Such "heating and snap cooling" favors the formation of shRNA monomers over multimers.

[0070] As used herein, the term "pharmaceutically acceptable" is a carrier, diluent, excipient, and/or salt that is compatible with the other ingredients of the formulation, and not deleterious to the recipient thereof. The active ingredient for administration may be present as a powder or as granules; as a solution, a suspension or an emulsion or as described elsewhere throughout the specification.

[0071] The phrase "pharmaceutically acceptable carrier", as used herein, means a pharmaceutically acceptable salt, solvent, suspending agent or vehicle for delivering a composition of the present disclosure to the animal or human. The carrier may be liquid, semisolid or solid, and is often synonymously used with diluent, excipient or salt. The phrase "pharmaceutically acceptable" means that an ingredient, excipient, carrier, diluent or component disclosed is one that is suitable for use with humans and/or animals without undue adverse side effects (such as toxicity, irritation, and allergic response) commensurate with a reasonable benefit/risk ratio. See Remington's Pharmaceutical Sciences 16.sup.th edition, Osol, A. Ed (1980) (incorporated herein by reference in its entirety).

[0072] As used herein, the term "operably-linked" refers to the association of nucleic acid sequences on a polynucleotide so that the function of one of the sequences is affected by another. For example, a regulatory DNA sequence is said

to be “operably linked to” a DNA sequence that codes for an RNA (“an RNA coding sequence” or “shRNA encoding sequence”) or a polypeptide if the two sequences are situated such that the regulatory DNA sequence affects expression of the coding DNA sequence (i.e., that the coding sequence or functional RNA is under the transcriptional control of the promoter). Coding sequences can be operably-linked to regulatory sequences in sense or antisense orientation. An RNA coding sequence refers to a nucleic acid that can serve as a template for synthesis of an RNA molecule such as an shRNA. Preferably, the RNA coding region is a DNA sequence.

[0073] As used herein, the term “promoter” refers to a nucleotide sequence, usually upstream (5') to its coding sequence, which directs and/or controls the expression of the coding sequence by providing the recognition for RNA polymerase and other factors required for proper transcription. “Promoter” includes a minimal promoter that is a short DNA sequence comprised of a TATA-box and other sequences that serve to specify the site of transcription initiation, to which regulatory elements are added for control of expression. “Promoter” also refers to a nucleotide sequence that includes a minimal promoter plus regulatory elements that is capable of controlling the expression of a coding sequence or functional RNA. This type of promoter sequence consists of proximal and more distal upstream elements, the latter elements often referred to as enhancers. Accordingly, an “enhancer” is a DNA sequence that stimulates promoter activity and may be an innate element of the promoter or a heterologous element inserted to enhance the level or tissue specificity of a promoter. It is capable of operating in both orientations (sense or antisense), and is capable of functioning even when moved either upstream or downstream from the promoter. Both enhancers and other upstream promoter elements bind sequence-specific DNA-binding proteins that mediate their effects. Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, or even be comprised of synthetic DNA segments. A promoter may also contain DNA sequences that are involved in the binding of protein factors that control the effectiveness of transcription initiation in response to physiological or developmental conditions. Any promoter known in the art which regulates the expression of the shRNA or RNA coding sequence is envisioned in the practice of the invention.

[0074] As used herein, the term “reporter element” or “marker” is meant a polynucleotide that encodes a polypeptide capable of being detected in a screening assay. Examples of polypeptides encoded by reporter elements include, but are not limited to, lacZ, GFP, luciferase, and chloramphenicol acetyltransferase. See, for example, U.S. Pat. No. 7,416,849. Many reporter elements and marker genes are known in the art and envisioned for use in the inventions disclosed herein.

[0075] As used herein, the term “RNA transcript” refers to the product resulting from RNA polymerase catalyzed transcription of a DNA sequence. “Messenger RNA transcript (mRNA)” refers to the RNA that is without introns and that can be translated into protein by the cell.

[0076] As used herein, the term “shRNA” (small hairpin RNA) refers to an RNA duplex wherein a portion of the RNA is part of a hairpin structure (shRNA). In addition to the duplex portion, the hairpin structure may contain a loop

portion positioned between the two sequences that form the duplex. The loop can vary in length. In some embodiments the loop is 5, 6, 7, 8, 9, 10, 11, 12 or 13 nucleotides in length. The hairpin structure can also contain 3' or 5' overhang portions. In some aspects, the overhang is a 3' or a 5' overhang 0, 1, 2, 3, 4 or 5 nucleotides in length. In one aspect of this invention, a nucleotide sequence in the vector serves as a template for the expression of a small hairpin RNA, comprising a sense region, a loop region and an antisense region. Following expression the sense and antisense regions form a duplex. It is this duplex, forming the shRNA, which hybridizes to, for example, the NOX2 mRNA and reduces expression of NADPH oxidase, lowering ROS and oxidative stress levels, fibrosis and AF.

[0077] As used herein, the term “knock-down” or “knock-down technology” refers to a technique of gene silencing in which the expression of a target gene or gene of interest is reduced as compared to the gene expression prior to the introduction of the siRNA, which can lead to the inhibition of production of the target gene product. “Double knock-down” is the knockdown of two genes. The term “reduced” is used herein to indicate that the target gene expression is lowered by 0.1-100%. For example, the expression may be reduced 0.5, 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or even 99%. The expression may be reduced by any amount (%) within those intervals, such as for example, 2-4, 11-14, 16-19, 21-24, 26-29, 31-34, 36-39, 41-44, 46-49, 51-54, 56-59, 61-64, 66-69, 71-74, 76-79, 81-84, 86-89, 91-94, 96, 97, 98 or 99. Knock-down of gene expression can be directed by the use of shRNAs.

[0078] As used herein, the term “treating” refers to ameliorating at least one symptom of, curing and/or preventing the development of a disease or disorder such as for example, but not limited to, cardiac fibrosis and AF.

[0079] As used herein, the term “vector” refers to any viral or non-viral vector, as well as any plasmid, cosmid, phage or binary vector in double or single stranded linear or circular form that may or may not be self transmissible or mobilizable, and that can transform prokaryotic or eukaryotic host cells either by integration into the cellular genome or which can exist extrachromosomally (e.g., autonomous replicating plasmid with an origin of replication). Any vector known in the art is envisioned for use in the practice of this invention.

[0080] The modal verb “may” refers to the preferred use or selection of one or more options or choices among the several described embodiments or features contained within the same. Where no options or choices are disclosed regarding a particular embodiment or feature contained in the same, the modal verb “may” refers to an affirmative act regarding how to make or use and aspect of a described embodiment or feature contained in the same, or a definitive decision to use a specific skill regarding a described embodiment or feature contained in the same. In this latter context, the modal verb “may” has the same meaning and connotation as the auxiliary verb “can.”

Compositions for Inhibiting NOX2 Gene Expression in Posterior Left Atrium (PLA) Tissue

[0081] In a one aspect, a pharmaceutical composition for treating oxidative stress in atrial fibrillation is provided. The pharmaceutical composition includes a small hairpin RNA (shRNA) directed against a NOX2 gene (“NOX2 shRNA”). The shRNA can be a unimolecular RNA that includes a sense sequence, a loop region, and an antisense sequence

(sometimes referred to as first and second regions, as noted above), which together form a hairpin loop structure. Preferably, the antisense and sense sequences are substantially complementary to one other (about 80% complementary or more), where in certain embodiments the antisense and sense sequences are 100% complementary to each other. In certain embodiments, the antisense and sense sequences are too short to be processed by Dicer, and hence act through an alternative pathway to that of longer double-stranded RNAs (e.g., shRNAs having antisense and sense sequences of about 16 to about 22 nucleotides in length, e.g., between 18 and 19 nucleotides in length (e.g., an sshRNA). Additionally, the antisense and sense sequences within a unimolecular RNA of the invention can be the same length, or differ in length by less than about 9 bases. The loop can be any length, with the preferred length being from 0 to 4 nucleotides in length or an equivalent length of non-nucleotidic linker, and more preferably 2 nucleotides or an equivalent length of non-nucleotidic linker (e.g., a non-nucleotide loop having a length equivalent to 2 nucleotides). In one embodiment, the loop is: 5'-UU-3' (rUrU) or 5'-tt-3', where "t" represents deoxythymidine (dTdT). Within any shRNA hairpin, a plurality of the nucleotides are ribonucleotides. In the case of a loop of zero nucleotides, the antisense sequence is linked directly to the sense sequence, with part of one or both strands forming the loop. In a preferred embodiment of a zero-nt loop shRNA, the antisense sequence is about 18 or 19 nt and the sense sequence is shorter than the antisense sequence, so that one end of the antisense sequence forms the loop.

[0082] A hairpin of representative shRNA's can be organized in either a left-handed (L) hairpin (i.e., 5'-antisense-loop-sense-3') or a right-handed (R) hairpin (i.e., 5'-sense-loop-antisense-3'). Furthermore, an shRNA may also contain overhangs at either the 5' or 3' end of either the sense sequence or the antisense sequence, depending upon the organization of the hairpin. Preferably, if there are any overhangs, they are on the 3' end of the hairpin and comprise between 1 to 6 bases. The presence of an overhang is preferred for R-type hairpins, in which case a 2-nt overhang is preferred, and a UU or tt overhang is most preferred.

[0083] Modifications can be added to enhance shRNA stability, functionality, and/or specificity and to minimize immunostimulatory properties. For example, the overhangs can be unmodified, or can contain one or more specificity or stabilizing modifications, such as a halogen or O-alkyl modification of the 2' position, or internucleotide modifications such as phosphorothioate modification. The overhangs can be ribonucleic acid, deoxyribonucleic acid, or a combination of ribonucleic acid and deoxyribonucleic acid.

[0084] In another non-limiting example of modifications that can be applied to left handed hairpins, 2'-O-methyl modifications (or other 2' modifications, including but not limited to other 2'-O-alkyl modifications) can be added to nucleotides at position 15, 17, or 19 from the 5' antisense terminus of the hairpin, or any two of those positions, or all three, as well as to the loop nucleotides and to every other nucleotide of the sense sequence except for nucleotides 9, 10 and 11 from the 5'-most nucleotide of the sense sequence (also called the 9.sup.th, 10.sup.th, and 11.sup.th nucleotides), which should have no modifications that block "slicing" activity. Any single modification or group of modifi-

cations described in the preceding sentence can be used alone or in combination with any other modification or group of modifications cited.

[0085] Ui-Tei, K. et al. (*Nucl. Acids Res.* (2008) 36 (22): 7100-7109) observed that the specificity of siRNAs can be increased by modifying the seed region of one or both strands. Such modifications are applicable to shRNA's of the present disclosure. In another non-limiting example of modifications that can be applied to hairpins, nt 1-6 of the antisense sequence and nt 14-19 of the sense sequence can be 2'-O-methylated to reduce off-target effects. In a preferred embodiment, only nt 1-6 are modified from 2'-OH to 2'-H or 2'-O-alky.

[0086] As the sense sequence of an shRNA can potentially enter RISC and compete with the antisense (targeting) strand, modifications that prevent sense sequence phosphorylation are valuable in minimizing off-target signatures. Thus, desirable chemical modifications that prevent phosphorylation of the 5' carbon of the 5'-most nucleotide of right-handed shRNA of the invention can include, but are not limited to, modifications that: (1) add a blocking group (e.g., a 5'-O-alkyl) to the 5' carbon; or (2) remove the 5'-hydroxyl group (e.g., 5'-deoxy nucleotides) (see, e.g., WO 2005/078094).

[0087] In addition to modifications that enhance specificity, modifications that enhance stability can also be added. In one embodiment, modifications comprising 2'-O-alkyl groups (or other 2' modifications) can be added to one or more, and preferably all, pyrimidines (e.g., C and/or U nucleotides) of the sense sequence. Modifications such as 2' F or 2'-O-alkyl of some or all of the Cs and Us of the sense sequence/region, respectively, or the loop structure, can enhance the stability of the shRNA molecules without appreciably altering target specific silencing. It should be noted that while these modifications enhance stability, it may be desirable to avoid the addition of these modification patterns to key positions in the hairpin in order to avoid disruption of RNAi (e.g., that interfere with "slicing" activity).

[0088] Additional stabilization modifications to the phosphate backbone may be included in the shRNAs in some embodiments of the present invention. For example, at least one phosphorothioate, phosphordithioate, and/or methylphosphonate may be substituted for the phosphate group at some or all 3' positions of nucleotides in the shRNA backbone, or any particular subset of nucleotides (e.g., any or all pyrimidines in the sense sequence of the oligonucleotide backbone), as well as in any overhangs, and/or loop structures present. These modifications may be used independently or in combination with the other modifications disclosed herein.

[0089] Description of modified shRNAs of interest can be found in the following references, both of which are incorporated herein by reference in their entirety: Q. Ge, H. Eves, A. Dallas, P. Kumar, J. Shorenstein, S. A. Kazakov, and B. H. Johnston (2010) Minimal-length short hairpin RNAs: The Relationship of Structure and RNAi Activity. *RNA* 16(1):106-17 (Epub Dec. 1, 2009); and Q. Ge, A. Dallas, H. Ilves, J. Shorenstein, M. A. Behlke, and B. H. Johnston (2010) Effects of Chemical Modification on the Potency, Serum Stability, and Immunostimulatory Properties of Short shRNAs. *RNA* 16(1):118-30 (Epub Nov. 30, 2009).

[0090] Modified shRNAs according to aspects of the present invention may include additional chemical modifications

for any of a variety of purposes, including 3' cap structures (e.g., an inverted deoxythymidine), detectable labels conjugated to one or more positions in the shRNA (e.g., fluorescent labels, mass labels, radioactive labels, etc.), or other conjugates that can enhance delivery, detection, function, specificity, or stability (e.g., amino acids, peptides, proteins, sugars, carbohydrates, lipids, polymers, nucleotides, polynucleotides, etc.). Combinations of additional chemical modifications may be employed as desired by the user.

[0091] Suitable NOX2 shRNAs include those nucleic acids ranging from about 20 nucleotides to about 80 nucleotides in length, wherein a portion of the nucleic acids have a double-stranded structural domain ranging from about 15 nucleotides to about 25 nucleotides in length. In some aspects, the shRNA can include modified bases or phosphodiester backbones to impart stability of the shRNA inside tissues and cells. An exemplary NOX2 shRNA includes SEQ ID NO:1 presented in Table 1.

TABLE 1

Exemplary NOX2 shRNA (SEQ ID NO: 1)
5' → 3' Nucleotide Sequence
TATCCATTCCAAGTCATAGG

[0092] shRNA: Synthesis

[0093] As is generally known in the art, commonly used oligonucleotides are oligomers or polymers of ribonucleic acid or deoxyribonucleic acid having a combination of naturally-occurring purine and pyrimidine bases, sugars and covalent linkages between nucleosides including a phosphate group in a phosphodiester linkage. However, it is noted that the term "oligonucleotides" also encompasses various non-naturally occurring mimetics and derivatives, i.e., modified forms, of naturally occurring oligonucleotides, as described herein.

[0094] shRNA molecules of the invention can be prepared by any method known in the art for the synthesis of DNA and RNA molecules. These include techniques for chemically synthesizing oligodeoxy-ribonucleotides and oligoribonucleotides well known in the art such as for example solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules can be generated by *in vitro* and *in vivo* transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors that incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines.

[0095] shRNA molecules can be chemically synthesized using appropriately protected ribonucleoside phosphoramidites and a conventional DNA/RNA synthesizer. Custom shRNA synthesis services are available from commercial vendors such as Ambion (Austin, Tex., USA) and Dharmacon Research (Lafayette, Colo., USA). See, for example, U.S. Pat. No. 7,410,944.

[0096] Various well-known modifications to the DNA molecules can be introduced as a means of increasing intracellular stability and half-life. Possible modifications include, but are not limited to, the addition of flanking sequences of ribo- or deoxy-nucleotides to the 5' and/or 3'

ends of the molecule or the use of phosphorothioate or 2'O-methyl rather than phosphodiester linkages within the oligodeoxyribonucleotide backbone. An antisense nucleic acid of the invention can be constructed using chemical synthesis or enzymatic ligation reactions using procedures known in the art. An antisense oligonucleotide can be chemically synthesized using naturally-occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the antisense and sense nucleic acids (e.g., phosphorothioate derivatives and acridine substituted nucleotides can be used).

[0097] The shRNA molecules of the invention can be various modified equivalents of the structures of any NOX2 shRNA. A "modified equivalent" means a modified form of a particular shRNA molecule having the same target-specificity (i.e., recognizing the same mRNA molecules that complement the unmodified particular shRNA molecule). Thus, a modified equivalent of an unmodified shRNA molecule can have modified ribonucleotides, that is, ribonucleotides that contain a modification in the chemical structure of an unmodified nucleotide base, sugar and/or phosphate (or phosphodiester linkage). See, for example, U.S. Pat. No. 7,410,944.

[0098] Preferably, modified shRNA molecules contain modified backbones or non-natural internucleoside linkages, e.g., modified phosphorous-containing backbones and non-phosphorous backbones such as morpholino backbones; siloxane, sulfide, sulfoxide, sulfone, sulfonate, sulfonamide, and sulfamate backbones; formacetyl and thioformacetyl backbones; alkene-containing backbones; methyleneimino and methylenehydrazino backbones; amide backbones, and the like. See, for example, U.S. Pat. No. 7,410,944.

[0099] Examples of modified phosphorous-containing backbones include, but are not limited to phosphorothioates, phosphorodithioates, chiral phosphorothioates, phosphotriesters, aminoalkylphosphotriesters, alkyl phosphonates, thionoalkylphosphonates, phosphinates, phosphoramidates, thionophosphoramidates, thionoalkylphosphotriesters, and boranophosphates and various salt forms thereof. See, for example, U.S. Pat. No. 7,410,944.

[0100] Examples of the non-phosphorous containing backbones described above are known in the art, e.g., U.S. Pat. No. 5,677,439, each of which is herein incorporated by reference. See, for example, U.S. Pat. No. 7,410,944.

[0101] Modified forms of shRNA compounds can also contain modified nucleosides (nucleoside analogs), i.e., modified purine or pyrimidine bases, e.g., 5-substituted pyrimidines, 6-azapyrimidines, pyridin-4-one, pyridin-2-one, phenyl, pseudouracil, 2,4,6-trimethoxy benzene, 3-methyl uracil, dihydrouridine, naphthyl, aminophenyl, 5-alkylcytidines (e.g., 5-methylcytidine), 5-alkyluridines (e.g., ribothymidine), 5-halouridine (e.g., 5-bromouridine) or 6-azapyrimidines or 6-alkylpyrimidines (e.g. 6-methyluridine), 2-thiouridine, 4-thiouridine, 5-(carboxyhydroxy methyl)uridine, 5'-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluridine, 5-methoxyaminomethyl-2-thiouridine, 5-methylaminomethyluridine, 5-methylcarbonylmethyl uridine, 5-methyloxyuridine, 5-methyl-2-thiouridine, 4-acetylcytidine, 3-methylcytidine, propyne, quenosine, wybutoxine, wybutoxine, beta-D-galactosylqueosine, N-2, N-6 and O-substituted purines, inosine, 1-methyladenosine, 1-methylinos-

ine, 2,2-dimethylguanosine, 2-methyladenosine, 2-methylguanosine, N6-methyladenosine, 7-methylguanosine, 2-methylthio-N-6-isopentenyl adenosine, beta-D-mannosylqueosine, uridine-5-oxyacetic acid, 2-thiocytidine, threonine derivatives, and the like. See, for example, U.S. Pat. No. 7,410,944.

[0102] In addition, modified shRNA compounds can also have substituted or modified sugar moieties, e.g., 2'-O-methoxyethyl sugar moieties. See, for example, U.S. Pat. No. 7,410,944.

[0103] Preferably, the 3' overhangs of the shRNAs of the present invention are modified to provide resistance to cellular nucleases. In one embodiment the 3' overhangs comprise 2'-deoxyribonucleotides.

[0104] Additional shRNA compounds targeted at different sites of the mRNA corresponding to NOX2. Additionally, to assist in the design of shRNAs for the efficient RNA interference (RNAi)-mediated silencing of any target gene, several shRNA supply companies maintain web-based design tools that utilize these general guidelines for "picking" shRNAs when presented with the mRNA or coding DNA sequence of the target gene. Examples of such tools can be found at the web sites of Dharmacon, Inc. (Lafayette, Colo.), Ambion, Inc. (Austin, Tex.). As an example, picking shRNAs involves choosing a site/sequence unique to the target gene (i.e., sequences that share no significant homology with genes other than the one being targeted), so that other genes are not inadvertently targeted by the same shRNA designed for this particular target sequence.

[0105] Another criterion to be considered is whether or not the target sequence includes a known polymorphic site. If so, shRNAs designed to target one particular allele may not effectively target another allele, since single base mismatches between the target sequence and its complementary strand in a given shRNA can greatly reduce the effectiveness of RNAi-induced by that shRNA. Given that target sequence and such design tools and design criteria, an ordinarily skilled artisan apprised of the present disclosure should be able to design and synthesized additional sihRNA compounds useful in reducing the mRNA level of NOX2.

[0106] shRNA: Administration

[0107] The present invention provides a composition of a polymer or excipient and one or more vectors encoding one or more shRNA molecules. The vector can be formulated into a pharmaceutical composition with suitable carriers and administered into a mammal using any suitable route of administration.

[0108] Because of this precision, side effects typically associated with traditional drugs can be reduced or eliminated. In addition, shRNA are relatively stable, and like antisense, they can also be modified to achieve improved pharmaceutical characteristics, such as increased stability, deliverability, and ease of manufacture. Moreover, because shRNA molecules take advantage of a natural cellular pathway, i.e., RNA interference, they are highly efficient in destroying targeted mRNA molecules. As a result, it is relatively easy to achieve a therapeutically effective concentration of an shRNA compound in a subject. See, for example, U.S. Pat. No. 7,410,944.

[0109] shRNA compounds may be administered to mammals by various methods through different routes. They can also be delivered directly to a particular organ or tissue by any suitable localized administration methods such as direct injection into a target tissue. Preferably, shRNA compounds

can be electroporated into cells following their injection directly into the target tissue. Alternatively, they may be delivered encapsulated in liposomes, by iontophoresis, or by incorporation into other vehicles such as hydrogels, cyclodextrins, biodegradable nanocapsules, and bioadhesive microspheres.

[0110] In vivo inhibition of specific gene expression by RNAi injected intravenously has been achieved in various organisms including mammals. See, for example, Song E. et al. "RNA interference targeting Fas protects mice from fulminant hepatitis," *Nature Medicine*, 9:347-351(2003). One route of administration of shRNA molecules of the invention includes direct injection of the vector at a desired tissue site, such as for example, into diseased or non-diseased cardiac tissue, into fibrotic heart tissue, such as fibrotic PLA tissue. Generally, however, NOX2 shRNAs or expression vectors encoding NOX2 shRNAs can be directly injected into any atrial tissue to effectively knock-down NOX2 protein expression, to inhibit NADPH oxidase enzyme activity, to effectively reduce ROS levels and oxidative stress in atrial tissues at the sites of injection, to reduce or altogether eliminate the presence of AF in a subject, or to reduce progression of fibrosis leading to increased AF presentation in a subject.

[0111] NAPDH oxidase (NOX2) is significantly elevated in the ventricle of heart failure patients, and is thought be a contributor to the heart failure state. Accordingly, NOX2 shRNAs or expression vectors encoding NOX2 shRNAs can be directly injected into ventricle tissue to effectively knock-down NOX2 protein expression, to inhibit NADPH oxidase enzyme activity, to effectively reduce ROS levels and oxidative stress in ventricle tissues at the sites of injection, to reduce or altogether eliminate progression of ventricle damage leading to increased HF presentation in a subject.

[0112] In one aspect of the invention, one or more vectors comprising one or more of shRNA of the invention can be readministered an unlimited number of times after a first administration at any time interval or intervals after the first administration.

[0113] shRNA: Pharmaceutical Compositions

[0114] The shRNA encoding nucleic acids of the present invention can be formulated in pharmaceutical compositions, which are prepared according to conventional pharmaceutical compounding techniques. See, e.g., Remington's Pharmaceutical Sciences, 18th Ed. (1990, Mack Publishing Co., Easton, Pa.). The pharmaceutical compositions of the invention comprise a therapeutically effective amount of the vector encoding shRNA. These compositions can comprise, in addition to the vector, a pharmaceutically acceptable excipient, carrier, buffer, stabilizer or other materials well known in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The carrier can take a wide variety of forms depending on the form of preparation desired for administration, e.g., intravenous, oral, intramuscular, subcutaneous, intrathecal, epineural or parenteral.

[0115] When the vectors of the invention are prepared for administration, they may be combined with a pharmaceutically acceptable carrier, diluent or excipient to form a pharmaceutical formulation, or unit dosage form. The total active ingredients in such formulations include from 0.1 to 99.9% by weight of the formulation.

[0116] In another aspect of the invention, the vectors of the invention can be suitably formulated and introduced into the

environment of the cell by any means that allows for a sufficient portion of the sample to enter the cell to induce gene silencing, if it is to occur. Many formulations for vectors are known in the art and can be used so long as the vectors gain entry to the target cells so that it can act.

[0117] For example, the vectors can be formulated in buffer solutions such as phosphate buffered saline solutions comprising liposomes, micellar structures, and capsids. The pharmaceutical formulations of the vectors of the invention can also take the form of an aqueous or anhydrous solution or dispersion, or alternatively the form of an emulsion or suspension. The pharmaceutical formulations of the vectors of the present invention may include, as optional ingredients, solubilizing or emulsifying agents, and salts of the type that are well-known in the art. Specific non-limiting examples of the carriers and/or diluents that are useful in the pharmaceutical formulations of the present invention include water and physiologically acceptable saline solutions. Other pharmaceutically acceptable carriers for preparing a composition for administration to an individual include, for example, solvents or vehicles such as glycols, glycerol, or injectable organic esters. A pharmaceutically acceptable carrier can contain physiologically acceptable compounds that act, for example, to stabilize or to increase the absorption of the shRNA encoding vector. Other physiologically acceptable carriers include, for example, carbohydrates, such as glucose, sucrose or dextrans, antioxidants, such as ascorbic acid or glutathione, chelating agents, low molecular weight proteins or other stabilizers or excipients, saline, dextrose solutions, fructose solutions, ethanol, or oils of animal, vegetative or synthetic origin. The carrier can also contain other ingredients, for example, preservatives.

[0118] It will be recognized that the choice of a pharmaceutically acceptable carrier, including a physiologically acceptable compound, depends, for example, on the route of administration of the composition. The composition containing the vectors can also contain a second reagent such as a diagnostic reagent, nutritional substance, toxin, or additional therapeutic agent. Many agents useful in the treatment of cardiac disease are known in the art and are envisioned for use in conjunction with the vectors of this invention.

[0119] Formulations of vectors with cationic lipids can be used to facilitate transfection of the vectors into cells. For example, cationic lipids, such as lipofectin, cationic glycerol derivatives, and polycationic molecules, such as polylysine, can be used. Suitable lipids include, for example, Oligofectamine and Lipofectamine (Life Technologies), which can be used according to the manufacturer's instructions.

[0120] Suitable amounts of vector must be introduced and these amounts can be empirically determined using standard methods. Typically, effective concentrations of individual vector species in the environment of a cell will be about 50 nanomolar or less 10 nanomolar or less, or compositions in which concentrations of about 1 nanomolar or less can be used. In other aspects, the methods utilize a concentration of about 200 picomolar or less and even a concentration of about 50 picomolar or less can be used in many circumstances. One of skill in the art can determine the effective concentration for any particular mammalian subject using standard methods.

[0121] The shRNA is preferably administered in a therapeutically effective amount. The actual amount administered, and the rate and time-course of administration, will depend on the nature and severity of the condition, disease

or disorder being treated. Prescription of treatment, for example, decisions on dosage, timing, etc., is within the responsibility of general practitioners or specialists, and typically takes account of the disorder, condition or disease to be treated, the condition of the individual mammalian subject, the site of delivery, the method of administration and other factors known to practitioners. Examples of techniques and protocols can be found in Remington's Pharmaceutical Sciences 18th Ed. (1990, Mack Publishing Co., Easton, Pa.).

[0122] Alternatively, targeting therapies can be used to deliver the shRNA encoding vectors more specifically to certain types of cell, by the use of targeting systems such as antibodies or cell specific ligands. Targeting can be desirable for a variety of reasons, e.g., if the agent is unacceptably toxic, or if it would otherwise require too high a dosage, or if it would not otherwise be able to enter the target cells.

[0123] shRNA: Gene Therapy

[0124] shRNA can also be delivered into mammalian cells, particularly human cells, by a gene therapy approach, using a DNA vector from which shRNA compounds in, e.g., small hairpin form (shRNA), can be transcribed directly. Recent studies have demonstrated that while double-stranded shRNAs are very effective at mediating RNAi, short, single-stranded, hairpin-shaped RNAs can also mediate RNAi, presumably because they fold into intramolecular duplexes that are processed into double-stranded shRNAs by cellular enzymes. This discovery has significant and far-reaching implications, since the production of such shRNAs can be readily achieved in vivo by transfecting cells or tissues with DNA vectors bearing short inverted repeats separated by a small number of (e.g., 3, 4, 5, 6, 7, 8, 9) nucleotides that direct the transcription of such small hairpin RNAs. Additionally, if mechanisms are included to direct the integration of the vector or a vector segment into the host-cell genome, or to ensure the stability of the transcription vector, the RNAi caused by the encoded shRNAs, can be made stable and heritable. Not only have such techniques been used to "knock down" the expression of specific genes in mammalian cells, but they have now been successfully employed to knock down the expression of exogenously expressed transgenes, as well as endogenous genes in the brain and liver of living mice.

[0125] Gene therapy is carried out according to generally accepted methods as are known in the art. See, for example, U.S. Pat. Nos. 5,837,492 and 5,800,998 and references cited therein. Vectors in the context of gene therapy are meant to include those polynucleotide sequences containing sequences sufficient to express a polynucleotide encoded therein. If the polynucleotide encodes an shRNA, expression will produce the antisense polynucleotide sequence. Thus, in this context, expression does not require that a protein product be synthesized. In addition to the shRNA encoded in the vector, the vector also contains a promoter functional in eukaryotic cells. The shRNA sequence is under control of this promoter. Suitable eukaryotic promoters include those described elsewhere herein and as are known in the art. The expression vector may also include sequences, such as selectable markers, reporter genes and other regulatory sequences conventionally used.

[0126] Accordingly, the amount of shRNA generated in situ is regulated by controlling such factors as the nature of the promoter used to direct transcription of the nucleic acid sequence, (i.e., whether the promoter is constitutive or regulatable, strong or weak) and the number of copies of the

nucleic acid sequence encoding a shRNA sequence that are in the cell. Exemplary promoters include those recognized by pol I, pol II and pol III. In some aspects, a preferred

promoter is a pol III promoter, such as the U6 pol III promoter. An exemplary vector for encoding an NOX2 shRNA is depicted in FIG. 4 and illustrated in Table 2.

TABLE 2

TABLE 2-continued

Expression vector encoding a NOX2 shRNA.

5' → 3' (nucleotide sequence) [SEQ ID NO: 2]

ggtgccgggaggcgccccgtggggcggtagtgtgggcctgttctgcggcgggtgtccgcatttcgcaagccccc
agcgacgtcgccagtcggctccctcggtgaccgaataccgacccctctccccagggggatccaccggagcttaccatg
accgagtagacaagcccacgggtgcgcctcgccaccggcgcacgcgtccccaggccgtacgcaccctcgccgcggctcgc
cgactaccggccacccgcgcacaccgtcgatccggaccggccacatcgagcgggtcaccgagctgcaagaactcttcc
cgccgcgtcggtcgacatcggaagggtgtgggtcgccgacgacggccgcgggtggggctggaccacgcggagac
gtcgaaggcgccgttcggccgcacccggccaaaggagccgcgtggttctggccaccgtcgccgtctcgccgcacc
gatggaaaggcctctggccgcacccggccaaaggagccgcgtggttctggccaccgtcgccgtctcgccgcacc
aggcgaagggtctgggcagccgcgtcggtctcccgagtgaggccggcgcggccgggtgcccgccttcctgg
accccgcccccaacctccccctacgcggctcggttaccgtcaccgcgcacgtcgaggtgcggcaaggacc
gcgcacccgtgtcgatgcggcaagccggcgtccgtacgcggcccccacgcaccgcgcgcggccaccgaaaggagcc
gaccccatgcacccgttacccatggtaagaccaatgacttacaggcagctgttagatcttagccactttaaaagaaaagg
ggactgaaagggtattactccaaacgaagacaatgtcttttgcgtactgggtctctgttagacc
ctgagcctggagctctggctaactaggaaacctactgttaagcctaataagcttgccttgcgtacttcaagtag
tgtgtgcggctgtgtgtactctgttaactagagatccctcagacccttttagtcaagtgtggaaatctctagact
agtagttcatgtcatcttattattcagttatataacttgcacaaagaaatgaatatcagagactgagaggaaactgttt
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gccaggccctactggcccttccggcttccggcttccggcttccggcttccggcttccggcttccggcttccggct
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ttccaaacttgc当地actcaacccttccggcttccggcttccggcttccggcttccggcttccggct
gttaaaaatgactgttttacttacggcttccggcttccggcttccggcttccggcttccggcttccggct
tcggggaaatgttgc当地acttccggcttccggcttccggcttccggcttccggcttccggct
cctgatataatgttccggcttccggcttccggcttccggcttccggcttccggcttccggct
ggccatccggcttccggcttccggcttccggcttccggcttccggcttccggcttccggct
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gcactttaaatgttccggcttccggcttccggcttccggcttccggcttccggct
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TABLE 2-continued

[0127] Kits

[0128] The subject invention also includes kits for inhibiting expression of a target gene in a cell, the kit including a chemically modified shRNA as described herein. A "kit" refers to any system for delivering materials or reagents for carrying out a method of the invention. In the context of reaction assays, such delivery systems include systems that allow for the storage, transport, or delivery of reaction reagents (e.g., chemically modified shRNA, culture medium, etc. in the appropriate containers) and/or supporting materials (e.g., buffers, written instructions for performing the assay, etc.) from one location to another. For example, kits include one or more enclosures (e.g., boxes) containing the relevant reaction reagents and/or supporting materials. Such contents may be delivered to the intended recipient together or separately. For example, a first container may contain a chemically modified shRNA for use in an assay, while a second container contains culture media RNA delivery agents (e.g., transfection reagents).

[0129] As noted above, the subject kits can further include instructions for using the components of the kit to practice the subject methods. The instructions for practicing the subject methods are generally recorded on a suitable recording medium. For example, the instructions may be printed on a substrate, such as paper or plastic, etc. As such, the instructions may be present in the kits as a package insert, in the labeling of the container of the kit or components

thereof (i.e., associated with the packaging or subpackaging) etc. In other embodiments, the instructions are present as an electronic storage data file present on a suitable computer readable storage medium, e.g. CD-ROM, diskette, etc. In yet other embodiments, the actual instructions are not present in the kit, but means for obtaining the instructions from a remote source, e.g. via the internet, are provided. An example of this embodiment is a kit that includes a web address where the instructions can be viewed and/or from which the instructions can be downloaded. As with the instructions, this means for obtaining the instructions is recorded on a suitable substrate.

[0130] In addition to the subject database, programming and instructions, the kits may also include one or more control reagents, e.g., non-chemically modified shRNA.

[0131] Methods

[0132] The pharmaceutical compositions have therapeutic efficacy in treating oxidative stress in a subject having atrial or ventricular arrhythmias, ventricular failure or heart failure. A pharmaceutical composition comprising a NOX2 shRNA has demonstrable activity in an art-accepted canine model for human AF. Experiments were performed with normal and HF animals to validate the utility of the canine model to study NOX2 knockdown assays and their effect on NOX2 expression and superoxide production. The HF animals produced superoxide at higher levels in the posterior left atrium (PLA) than in the left atrial appendage (LAA) as

compared to normal animals (FIG. 2A). NADPH oxidase is a major source of superoxide generation in PLA from HF animals compared to normal animals (FIG. 2B) depicts contribution of O₂⁻ production in PLA for NADPH oxidase (blue bars), Mitochondrial ROS (red bars), NOS (green bars) and Xanthine oxidase (purple bars). Furthermore, HF animals display higher levels of NOX2 polypeptide subunit (gp91) as compared to normal animals (FIGS. 3A, B).

[0133] A plasmid encoding either NOX2 shRNA (FIG. 4 and SEQ ID NOs: 1 and 2) or a control RNA (lac Z) was introduced into PLA tissues of HF animals and the effects on AF were evaluated. The duration of AF (seconds) is decreased in plasmid encoding NOX2 shRNA-treated animals ("NOX2 shRNA") compared with lacZ-transfected control animals ("HF (LacZ Control)") (FIG. 5). Moreover, expression of ox-CAMKII in the PLA of HF dogs transfected with NOX2 shRNA (FIG. 6B) is lower than expression of ox-CAMKII in the PLA of HF dogs (FIG. 6A), which is consistent with NOX2 shRNA knocking down NOX2 expression and lowering oxidative stress in atrial tissues.

[0134] Electrophysiological measurements were recorded for animals following 3 weeks of rapid atrial pacing. Effective Refractory Periods (ERPs) were increased significantly following pacing conditioning; however, ERPs did not change significantly as a function of NOX2 knockdown using NOX2 shRNA (FIG. 7). Conduction was less heterogeneous in PLA transfected with plasmid encoding NOX2 shRNA compared to PLA transfected with control plasmid (FIGS. 8A, B) and NOX2 shRNA abolished significant change noted in the conductive inhomogeneity index (FIGS. 8C, D).

[0135] NOX2 mRNA and protein is reduced in PLA transfected with plasmid encoding NOX2 shRNA compared to PLA transfected with control plasmid (FIGS. 9A, B). Similarly, the relative contribution of superoxide production attributed to NADPH oxidase is lower in PLA transfected with plasmid encoding NOX2 shRNA compared to PLA transfected with control plasmid (FIG. 10).

[0136] In addition to using traditional heart monitoring methodologies to detect the presence and absence of AF as a function of NOX2 shRNA therapy, AF EGM characteristics can be systematically assessed to evaluate structural remodeling of atrial tissue in candidate subjects as well as therapeutic efficacy of the pharmaceutical compositions for inhibiting oxidative stress for subjects suffering from chronic AF. Subjects who suffer from chronic AF have structural remodeled atrial tissue with fibrosis. In this regard, fibrosis was reduced in animals whose PLA was transfected with plasmid encoding NOX2 shRNA compared to control animals (FIG. 11A-C).

[0137] Application of EGM-guided identification and monitoring of fibrosis-associated structural remodeling of atrial tissue in AF and HF is summarized by related patent application publications by the inventors, such as application entitled "INHIBITION OF FIBROSIS AND AF BY TGF-BETA INHIBITION IN THE POSTERIOR LEFT ATRIUM (PLA)" to Rishi Arora, bearing Ser. No. 13/890, 116, filed May 8, 2013, now U.S. Patent Application Publication US 2014-0037545 A1 and application entitled "USING INTRACARDIAC F.T ECTROGRAMS TO PREDICT LOCATION OF FIBROSIS AND AUTONOMIC NERVES IN THE HEART" to Rishi Arora et al, bearing Ser. No. 13/890,112, filed May 8, 2013, now U.S. Patent Application Publication US 2013-0324869A1.

[0138] In another model, electrical remodeling of atrial tissues can lead to spontaneous AF. Such a model reflects AF in a subject who otherwise might not have significant structural remodeling of atrial tissue due to fibrosis (for example, young subjects). Pacemakers can be used to generate rapid tachypacing in the canine model of electrical remodeling of atrial tissue. Heart monitors (for example, ECG or EKG patterns) can be used to detect AF development as well as the efficacy of NOX2 shRNA treatment efficacy in atrial tissues by measuring the decline or absence of AF following treatment. In this regard, the onset of AF episodes was blocked in animals whose PLA was transfected with plasmid encoding NOX2 shRNA, as compared to normal animals following rapid atrial pacing of the animals (FIG. 12). Applicability to other NOX2 inhibitors.

[0139] The results of the NOX2 shRNA studies demonstrate the feasibility of a general strategy to inhibit NADPH oxidase by targeting NOX2 using NOX2 inhibitors. Such inhibitor agents include oligonucleotide-based compounds that target the NOX2 mRNA or protein, such as RNAi molecules and shRNAs directed against NOX2 mRNA and oligonucleotide-based aptamers directed against the NOX2 polypeptide. Furthermore, small molecule organic compounds having anti-NADPH oxidase activity by specifically binding to or otherwise interfering with NOX2 protein functionality can also reduce NADPH oxidase-mediated superoxide production and oxidative stress in AF.

[0140] In some embodiments, NOX2 inhibitors comprise any suitable bioactive molecules (e.g. a molecule capable of inhibiting the function of NADPH oxidase). In some embodiments, a NOX2 inhibitor comprises a macromolecule, polymer, a molecular complex, protein, peptide, polypeptide, nucleic acid, carbohydrate, small molecule, etc.

[0141] In some embodiments, a NOX2 inhibitor is a NOX2 inhibitory peptide. In some embodiments, the present invention provides peptides of any suitable amino acid sequence capable of inhibiting one or more alleles of NOX2. In some embodiments, peptides provided by or encoded by the compositions of embodiments of the present invention may comprise any arrangement of any standard amino acids (e.g. alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine) or non-standard amino acids (e.g. D-amino acids, chemically or biologically produced derivatives of common amino acids, selenocysteine, pyrrollysine, lanthionine, 2-aminoisobutyric acid, dehydroalanine, etc.). In some embodiments, NOX2 inhibitory peptides are inhibitors to NOX2.

[0142] In some embodiments, NOX2 inhibitory peptides are provided to a subject as isolated or purified peptides. In some embodiments, NOX2 inhibitory peptides are provided to a subject as nucleic acid molecules that encode such peptides. In some embodiments, peptides are optimized to enhance cell penetration (e.g. sequence optimization, sequence tag, tagged with a small molecule, etc.).

[0143] In some embodiments, a NOX2 inhibitor is provided from an isolated nucleic acid comprising a minigene, wherein said minigene encodes a modified NOX2 peptide, wherein the peptide blocks the site of interaction between NOX2 and NOX2 binding partners in a cell, such as a human cell. In addition, the minigene can further comprise one or more of a promoter, a ribosomal binding site, a translation initiation codon, and a translation termination codon. In

some embodiments, the minigene encodes a modified NOX2 peptide having one of the following general formulas: MGX, MX, and MZX, wherein M is a methionine amino acid residue, wherein G is a glycine amino acid residue, wherein Z is an amino acid residue other than a glycine amino acid residue, and wherein X is a NOX2 peptide which comprises an amino acid sequence from the NOX2 subunit, and has the property of binding a NOX2 binding partner. In this embodiment, X can comprise from at least about three contiguous amino acids to at least about 54 contiguous amino acids, from at least about three contiguous amino acids to at least about eleven contiguous amino acids, and at least about eleven contiguous amino acids. In one embodiment, X comprises the seven contiguous amino acid residues of the NOX2 subunit.

[0144] In some embodiments, the NOX2 inhibitor is provided as an isolated or purified polypeptide. In some embodiments, the peptide has a general formula selected from the group consisting of MGX, MX, and MZX, wherein M is a methionine amino acid residue, wherein G is a glycine amino acid residue, wherein Z is an amino acid residue other than a glycine amino acid residue, and wherein X is a NOX2-derived peptide which comprises an amino acid sequence of the NOX2 subunit, and has the property of binding a NOX2 binding partner. In this embodiment, X can comprise from at least about three contiguous amino acids to at least about 54 contiguous amino acids, from at least about three contiguous amino acids to at least about eleven contiguous amino acids, and at least about eleven contiguous amino acids. In one embodiment, X comprises the seven contiguous amino acid residues of the NOX2 subunit.

[0145] In some embodiments, the present invention provides methods of inhibiting a NOX2-mediated signaling event in a cell or tissue. These methods comprise administering to a cell or tissue, preferably a human cell or tissue, one of a modified NOX2 peptide and an isolated nucleic acid comprising a minigene which encodes a modified NOX2 peptide, whereby following the administration, the NOX2 peptide inhibits the NOX2-mediated signaling event in the cell or tissue.

[0146] In some embodiments, a NOX2 inhibitor comprises a small molecule. In some embodiments, the present invention provides a small molecule inhibitor of NOX2. In some embodiments, the present invention provides a small molecule drug or pharmaceutical compound configured to or capable of inhibiting NOX2 activity, function expression, or the like.

[0147] In some embodiments, the present invention provides RNAi molecules (e.g., that alter NOX2 expression) as a NOX2 inhibitor. In some embodiments, the present invention targets the expression of NOX2 genes using nucleic acid based therapies. For example, in some embodiments, the present invention employs compositions comprising oligomeric antisense or RNAi compounds, particularly oligonucleotides, for use in modulating the function of nucleic acid molecules encoding NOX2 genes, ultimately modulating the amount of NOX2 protein expressed. In some embodiments, RNAi is utilized to inhibit NOX2 gene function. RNAi represents an evolutionary conserved cellular defense for controlling the expression of foreign genes in most eukaryotes, including humans. RNAi is typically triggered by double-stranded RNA (dsRNA) and causes sequence-specific mRNA degradation of single-stranded target RNAs homologous in response to dsRNA. The media-

tors of mRNA degradation are small interfering RNA duplexes (siRNAs), which are normally produced from long dsRNA by enzymatic cleavage in the cell. siRNAs are generally approximately twenty-one nucleotides in length (e.g. 21-23 nucleotides in length), and have a base-paired structure characterized by two nucleotide 3'-overhangs. Following the introduction of a small RNA, or RNAi, into the cell, it is believed the sequence is delivered to an enzyme complex called RISC (RNA-induced silencing complex). RISC recognizes the target and cleaves it with an endonuclease. It is noted that if larger RNA sequences are delivered to a cell, RNase III enzyme (Dicer) converts longer dsRNA into 21-23 nt ds siRNA fragments.

[0148] The transfection of siRNAs into animal cells results in the potent, long-lasting post-transcriptional silencing of specific genes (Caplen et al, Proc Natl Acad Sci U.S.A. 2001; 98: 9742-7; Elbashir et al., Nature. 2001; 411:494-8; Elbashir et al., Genes Dev. 2001; 15: 188-200; and Elbashir et al., EMBO J. 2001; 20: 6877-88, all of which are herein incorporated by reference). Methods and compositions for performing RNAi with siRNAs are described, for example, in U.S. Pat. No. 6,506,559, herein incorporated by reference.

[0149] siRNAs are extraordinarily effective at lowering the amounts of targeted RNA, and by extension proteins, frequently to undetectable levels. The silencing effect can last several months, and is extraordinarily specific, because one nucleotide mismatch between the target RNA and the central region of the siRNA is frequently sufficient to prevent silencing (Brummelkamp et al, Science 2002; 296: 550-3; and Holen et al, Nucleic Acids Res. 2002; 30:1757-66, both of which are herein incorporated by reference).

[0150] In some embodiments, NOX2 expression is modulated using antisense compounds that specifically hybridize with one or more nucleic acids encoding NOX2. The specific hybridization of an oligomeric compound with its target nucleic acid interferes with the normal function of the nucleic acid. This modulation of function of a target nucleic acid by compounds that specifically hybridize to it is generally referred to as "antisense."

[0151] In some embodiments, the present invention contemplates the use of any genetic manipulation for use in modulating the expression of NOX2 genes. Examples of genetic manipulation include, but are not limited to, gene knockout (e.g., removing the NOX2 gene from the chromosome using, for example, recombination), expression of antisense constructs with or without inducible promoters, and the like. Delivery of nucleic acid construct to cells in vitro or in vivo may be conducted using any suitable method. A suitable method is one that introduces the nucleic acid construct into the cell such that the desired event occurs (e.g., expression of an antisense construct). Genetic therapy may also be used to deliver siRNA or other interfering molecules that are expressed in vivo (e.g., upon stimulation by an inducible promoter).

[0152] In some embodiments, the present invention provides antibodies that target NOX2 protein. Any suitable antibody (e.g., monoclonal, polyclonal, or synthetic) may be utilized in the therapeutic methods disclosed herein. In preferred embodiments, the antibodies are humanized antibodies. Methods for humanizing antibodies are well known in the art (See e.g., U.S. Pat. Nos. 6,180,370, 5,585,089, 6,054,297, and 5,565,332; each of which is herein incorporated by reference).

[0153] In some embodiments, the present invention provides methods of enhancing entry of a NOX2 inhibitor into cells or tissue. In some embodiments, the present invention provides administering a NOX2 inhibitor in conjunction with electroporation, electropermeabilization, or sonoporation. In some embodiments, the present invention provides administering a NOX2 inhibitor in conjunction with electroporation. In some embodiments, the present invention provides co-injection/electroporation of the tissue of a subject. In some embodiments, the present invention provides administering a NOX2 inhibitor prior to, simultaneously with, and/or following electroporation. In some embodiments, electroporation provides a method of delivering pharmaceuticals or nucleic acids (e.g. DNA) into cells. In some embodiments, tissue electrically stimulated at the same time or shortly after pharmaceutical or DNA is applied (e.g. NOX2 inhibitor). In some embodiments, electroporation increases cell permeability. The permeability or the pores are large enough to allow the pharmaceuticals and/or DNA to gain access to the cells. In some embodiments, the pores in the cell membrane close and the cell once again becomes impermeable or less permeable. Devices for co-injection/electroporation are known in the art (U.S. Pat. No. 7,328,064, herein incorporated by reference in its entirety).

[0154] Furthermore, though the canine model utilized PLA as model atrial tissue, the approach is applicable to all atrial tissues. In some embodiments, the present invention provides compositions and methods to treat or prevent conditions and/or diseases of the heart (e.g. rhythm disturbances (e.g. atrial fibrillation)). In some embodiments, the present invention provides treatment or prevention of a heart disease or condition selected from the list of aortic dissection, cardiac arrhythmia (e.g. atrial cardiac arrhythmia (e.g. premature atrial contractions, wandering atrial pacemaker, multifocal atrial tachycardia, atrial flutter, atrial fibrillation, etc.), junctional arrhythmias (e.g. supraventricular tachycardia, AV nodal reentrant tachycardia, paroxysmal supraventricular tachycardia, junctional rhythm, junctional tachycardia, premature junctional complex, etc.), atrio-ventricular arrhythmias, ventricular arrhythmias (e.g. premature ventricular contractions, accelerated idioventricular rhythm, monomorphic ventricular tachycardia, polymorphic ventricular tachycardia, ventricular fibrillation, etc.), etc.), congenital heart disease, myocardial infarction, dilated cardiomyopathy, hypertrophic cardiomyopathy, aortic regurgitation, aortic stenosis, mitral regurgitation, mitral stenosis, Ellis-van Creveld syndrome, familial hypertrophic cardiomyopathy, Holt-Orams Syndrome, Marfan Syndrome, Ward-Romano Syndrome, and/or similar diseases and conditions.

[0155] In some embodiments, the present invention provides compositions and methods to treat atrial fibrillation. Atrial fibrillation is the commonest rhythm disturbance of the heart. The posterior left atrium and pulmonary veins have been shown to play an important role in the genesis of atrial fibrillation. More recent studies demonstrate a role for the autonomic nervous system, especially the parasympathetic nervous system, in the genesis of atrial fibrillation from the posterior left atrium. Current therapies to manage atrial fibrillation remain ineffective, while novel links, including autonomic activity described here, provide for beneficial treatment options. Work conducted during the development of embodiments of the present invention shows that selective disruption of autonomic pathways in the

posterior left atrium can significantly modify the ability to the heart to sustain atrial fibrillation. In particular, embodiments of the present invention treat atrial fibrillation by administration of NOX2 inhibitors. An understanding of the mechanism of action is not necessary to practice the present invention and the present invention is not limited to any particular mechanism of action. The present invention also provides compositions and methods for researching atrial fibrillation, including screening for compounds useful in treating, prevent, or reducing signs or symptoms associate with atrial fibrillation.

EXAMPLES

[0156] The invention will be more fully understood upon consideration of the following non-limiting examples, which are offered for purposes of illustration, not limitation.

Example 1

HF Canine Model of Structural Remodeling and AF

[0157] Dog Protocols.

[0158] Purpose-bred hound dogs (weight range: 25-35 kg; age rage: 1-3 years) used in this study were maintained in accordance to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996) as approved by the Animal Care and Use Committee of Northwestern University. Before undergoing the procedures listed below, all animals were sedated with diazepam 0.22 mg/kg IM and were induced with propofol (3-7 mg/kg IV). After animals were unresponsive, they were intubated and positive-pressure ventilated, and anesthesia was maintained with isoflurane 0.5-1.5%. The surgical field was scrubbed with chlorhexidine and isopropyl alcohol, sprayed with betadine solution, and draped with sterile towels and sheets. The chest was then opened via a left lateral thoracotomy.

[0159] Eleven hounds were randomized into 2 groups: 1) control (n=7) expressing LacZ and 2) gene therapy with a plasmid expressing NOX2 shRNA (n=4). During an initial procedure, a left lateral thoracotomy was performed and the pericardium was incised; animals underwent invasive electrophysiology study (EPS), gene injection, echocardiogram and epicardial implantation of a left ventricular pacemaker. After animals were allowed to recover for 3-5 days, ventricular tachypacing was initiated at 240 bpm. Clinical status assessment and pacing was verified daily. Three weeks after the initial procedure, animals underwent terminal echocardiogram, FPS and cardiac extraction for histology and molecular studies.

[0160] Initial Open Chest Electrophysiological Mapping

[0161] Effective Refractory Periods (ERPs). ERPs were obtained using two rectangular plaques epicardially positioned on the PLA and LAA. Each plaque had 21 electrodes each (7×3 electrodes, inter-electrode distance=5 mm). A Bloom stimulator interfaced with a GE Prucka Cardiolab system (GE Healthcare, Waukesha, Wis.) were used to perform programmed stimulation. ERPs were measured by delivering an 8 pulse drive train at 400 ms coupled to a premature stimulus. The coupling interval of the premature stimulus was decreased in 10 ms increments, and the ERP was defined as the longest coupling interval that fails to conduct. ERPs were obtained from five distributed sites in

the both the PLA and LAA. FIG. 7 depicts changes in ERP in plasmid encoding NOX2 shRNA-treated animals (NOX2 shRNA) compared with lacZ-transfected control animals (HF) for baseline (black bars) and post-pacing (grey bars) conditioned animals.

[0162] AF inducibility. AF was induced with burst pacing at maximum output using cycle lengths of 180 ms to 100 ms with 10 ms decrements for 10 seconds for each cycle length. Current was set at four times threshold for capture. AF induction was defined as episodes lasting more than 30 seconds.

[0163] Activation Mapping. High density activation mapping was performed using a UNEMAP mapping system (Univ. of Auckland, Auckland, New Zealand). A triangular plaque containing 130 electrodes (inter-electrode distance of 2.5 mm) were used to record 117 bipolar EGMs at a 1 kHz sampling rate. Mapping was performed sequentially in the LAA and in two adjacent sites in the PLA. At each site, 10 second recordings were made during sinus rhythm and pacing with cycle lengths of 400, 300, and 200 ms. Pacing was performed in LAA when recording from the PLA and vice versa.

[0164] Data analysis with Conduction Inhomogeneity. MATLAB (Mathworks, Natick, Mass.) was used for all offline signal analysis in this study. Conduction inhomogeneity analysis was performed using the high density UNEMAP recordings obtained during sinus rhythm and pacing. The bipolar electrograms were highpass filtered at 30 Hz, rectified, and then lowpass filtered at 20 Hz. The times of the filtering peaks were considered the activation time for that activation. Conduction inhomogeneity was calculated as described by Lammers W J, Schalij M J, Kirchhof C J, Allessie M A. Quantification of spatial inhomogeneity in conduction and initiation of reentrant atrial arrhythmias. Am J Physiol 1990; 259:H1254-63. Total activation time measuring the amount of time to activate the entire area of the plaque was also measured. Total activation time measuring the amount of time to activate the entire area of the plaque was also measured. Exemplary results of these studies are shown in FIG. 8A-D.

[0165] Gene Injection/Transfer.

[0166] After the completion of the initial EPS, 10 mg of plasmid DNA [control plasmid (pUBc-lacZ) or NOX2 shRNA-encoded plasmid expression vector under the control of the U6 pol III promoter (FIG. 4)] (0.1 mg/each site) was directly injected transmurally in the PLA with a 27-gauge needle. Electric pulses were delivered to the myocardium through the electrodes spaced 1 cm apart (Genetrodes, BTX). Immediately after the injection, 8 electrical pulses (amplitude, 200 V; duration, 10 ms; intervals, 1 sec) were delivered with a square-wave electroporation generator (ECM 830, BTX, Harvard Apparatus).

[0167] HF Induction.

[0168] In 11 dogs, HF was induced by 3 to 4 weeks of right ventricular tachypacing (240 beats per minute) by an implanted pacemaker. In all dogs, a pacemaker was placed on the ventricle via an epicardial approach (that is, via a left lateral thoracotomy). Left ventricular function was assessed during pacing by serial echocardiograms (data not shown). HF was confirmed after 3 to 4 weeks of pacing.

[0169] Terminal surgery and open chest electrophysiology mapping. At the terminal study, a left lateral thoracotomy was performed. Low density and high density mapping protocols were used. With low density mapping, the poste-

rior left atrium (PLA), left superior pulmonary vein (PV), and left atrial appendage could be mapped simultaneously. The PLA and LAA were mapped using two rectangular plaques containing 21 electrodes each (7×3 electrodes, inter-electrode distance=5 mm) from which 18 bipolar EGMs were recorded. FIG. 3A shows the schematics of the plaques. The signals from the low density plaques were recorded and stored at a 977 Hz sampling rate with the GE Prucka Cardiolab system (GE Healthcare, Waukesha, Wis.).

[0170] High density mapping was performed in all dogs for more detailed electrophysiological analysis, including assess of conduction heterogeneity (inhomogeneity). Mapping was performed sequentially in the PLA and LAA with a triangular plaque containing 130 electrodes (inter-electrode distance of 2.5 mm) from which 117 bipolar electrograms (EGMs) were recorded. The UNEMAP mapping system (Univ. of Auckland, Auckland, New Zealand) was used for recording and storing the EGMs at a 1 kHz sampling rate. Even though we did not separately map the PVs during high-density mapping (owing to the relatively large surface area of the high density plaques, it was technically challenging to cover the PVs, which have a circular and uneven surface), the high-density plaque did straddle the proximal PVs during PLA mapping.

Example 2

Rapid Atrial Pacing (RAP) Canine Model of Electrical Remodeling and Spontaneous AF

[0171] A transvenous pacemaker was placed in the right atrium (RA) via a jugular venous approach. One week later, the animal underwent a left lateral thoracotomy. Open chest electrophysiological mapping was performed as follows:

[0172] Initial Open Chest Electrophysiological Mapping

[0173] Effective Refractory Periods (ERPs). ERPs were obtained using two rectangular, 21-electrode plaques epicardially positioned on the PLA and LAA. ERPs were obtained from five evenly distributed sites in the both the PLA and LAA.

[0174] AF inducibility. AF was induced with burst pacing. AF was defined as episodes lasting more than 3 seconds. Sustained AF was defined as AF>60 seconds.

[0175] Activation Mapping. High density activation mapping was performed using the UNEMAP mapping system (Univ. of Auckland, Auckland, New Zealand). A triangular plaque containing 130 electrodes (inter-electrode distance of 2.5 mm) were used to record 117 bipolar EGMs at a 1 kHz sampling rate. Mapping was performed sequentially in the LAA and in two adjacent sites in the PLA. At each site, 10 second recordings were made during sinus rhythm and pacing with cycle lengths of 400, 300, and 200 ms.

[0176] Gene injection/transfer. After the completion of the initial electrophysiological study, 10 mg of plasmid [control plasmid (pUBc-lacZ) or NOX2 shRNA-encoded plasmid expression vector under the control of the U6 pol III promoter (FIG. 4)] (diluted in 5 ml sterile saline) was injected sub-epicardially in the PLA with a 27-gauge needle; 5-6 injections (approximately 1 ml each) were used to cover the entire PLA. After gene injection, electroporation was performed at each site of gene injection (200 V; duration, 10 ms; intervals, 1 sec).

[0177] Rapid atrial pacing. After the initial open-chest mapping study and gene injection, the chest was closed and the animal allowed to recover for 1 week. Rapid atrial

pacing (at 600 beats/min) was then started, and continued for the next 3 weeks. FIG. 12 illustrates that dog recipients of NOX2 shRNA-encoded plasmid expression vector injected into PLA tissue did not develop spontaneous AF following rapid atrial pacing.

[0178] Serial testing for sustained AF. Every 24-48 hours, pacing was stopped for 30-60 minutes, in order to assess for onset of AF and for duration of the induced AF.

[0179] Terminal surgery and open chest electrophysiology mapping. After 3 weeks of rapid atrial pacing (RAP), repeat open chest electrophysiological mapping was performed. If the animal reverted to sinus rhythm, atrial effective refractory periods were assessed. AF electrograms were also recorded.

[0180] After the electrophysiological study was completed, the heart was removed and the atria snap frozen. The atria was subjected to further analysis to assess for gene expression, expression of key signaling molecules etc.

Example 3

Histology and Tissue Analysis.

[0181] The histologic analysis described below was performed on atrial tissues from untreated and treated dogs receiving plasmids expressing NOX2 shRNA or an inactive control RNA (lac Z) were analyzed.

[0182] Tissue Sample Preparation. In the animals undergoing high density mapping, immediately following the in vivo electrophysiological study, the heart was promptly excised out of the chest and immersed in ice-cold cardioplegia as previously described by us. After marking the exact orientation of the high density plaques, tissue samples were taken from the PLA and LAA regions of the left atrium and snap frozen in liquid nitrogen. Samples were saved in the exact orientation in which high density mapping had been performed. All samples were initially saved at -80° C. The oriented tissue samples were frozen in Tissue-Tek OCT (Optimal Cutting Temperature) compound at -80° C.

[0183] For paraffinization, the tissue was thawed and a quick wash given to dean off all the OCT, Using a PCF LEICA 1050 Tissue Processor, the tissue was embedded in paraffin. The tissue processor uses 10% NBF(Neutral Buffered Formalin) for fixing and the tissue dehydration is performed with incremental concentrations of Ethanol(ETOH). ETOH is exchanged with xylene and finally xylene is exchanged with paraffin at 58° C. Then tissue is embedded in a paraffin block.

[0184] Masson's Trichrome staining. Tissue sections were cut 4 µm apart. Paraffin was removed by placing the tissue section in histology grade xylene for two minutes and the process was repeated four times changing xylene solution after every two minutes. Finally, the xylene was washed away with ETOH for one minute in absolute ETOH, then again for one more minute with fresh absolute ETOH, followed by wash in 95% ETOH for 30 seconds, and subsequently in 70% ETOH for 45 seconds. ETOH was then washed with water for one minute. The tissue section was then ready for staining. The section was treated with Bouin's mordant at room temperature overnight. The following day the tissue section was rinsed in running water to remove excess yellow. The tissue section was stained in Weigert's Solution for 7 minutes. Next, it was dipped once in 1% acid alcohol and immediately rinsed. The section was then stained in Beirich Scarlet-Acid fuchsin for 2 minutes,

followed by a rinse in distilled water. Subsequently, the tissue section was stained in phosphomolybdic-phosphotungstic acid solution for 6 minutes, followed by another rinse in distilled water. The issue section was then stained in Aniline Blue solution for 5 minutes, followed by another rinse in distilled water. Immediately, the tissue was dipped once in 1%Glacial acetic acid and quickly rinsed. The tissue section was then dehydrated in twice in each concentration of 95% and 100% of ETOH, which was later exchanged with xylene. A coverslip was finally placed on the tissue section for microscope examination. Examples of results of such assays are illustrated in FIG. 11A-C.

[0185] ox-CAMKII immunofluorescence. Tissue samples were fixed and stained with anti-ox-CAMKII antibody. Exemplary results of such assays are illustrated in FIGS. 6A, B.

[0186] Real-time PCR. Frozen tissue samples were frozen crushed and homogenized. Total RNA was isolated using Trizol Reagent (Life Technologies, 15596-026). Contaminating DNA was removed using DNA-free DNA Removal Kit (Life Technologies, AM1906). cDNA was synthesized from 0.5 µg of total RNA with TaqMan reverse transcription reagents SuperScript VILO (Life Technologies, #11755050) and mixed with TaqMan Fast Advanced Master Mix (Life Technologies, #4444965). Quantitative real-time PCR (qRT-PCR) was carried out using Applied Biosystems® 7500 Fast Real-Time PCR System (Life Technologies). Relative mRNA levels were calculated by the (type of software) after normalization of each experimental sample to GAPDH levels. An example of results of such assays is illustrated in FIG. 9A.

[0187] Western Blot Analysis. The atrial tissue was snap frozen in liquid nitrogen, homogenized, and separated into membrane and cytosolic fractions according to the procedures of the Mem-Per Plus Membrane Protein Extraction Kit (Thermo-Scientific, #89842) or T-Per Tissue Protein Extraction Reagent (Thermo-Scientific, 78510) for total protein. We added Halt Protease & Phosphatase Inhibitor Cocktail (Thermo-Scientific, #78446) to all buffers. Protein concentrations were determined using Pierce BCA Protein Assay Kit (Thermo-Scientific, #23227). Proteins were fractionated by SDS-PAGE, transferred to polyvinyl difluoride (PVDF) membrane, blocked with 5% BSA, and blotted with the appropriate primary and secondary antibodies. Examples of results of such assays are illustrated in FIG. 3 and FIG. 9B.

[0188] Measurement of NADPH-dependent superoxide production by chemiluminescence. 100 mg frozen tissue samples were crushed and rotor homogenized (Biospec Products Inc, Tissue-Tearor) with protease inhibitor (Halt protease and phosphatase inhibitor cocktail, Thermo-Scientific, #1861282). Protein concentrations were determined using Pierce BCA Protein Assay Kit (Thermo-Scientific, #23227). Lucigenin (5 µmol/L) (Enzo Life Sciences, ALX-620-061-M050) and NADPH (100 µmol/L) (Calbiochem, #181973) were each added in the presence and absence of apocynin (Santa Cruz Biotechnology, #sc-203321) (an inhibitor of NADPH oxidase) and mito-TEMPO (Santa Cruz Biotechnology, #sc-221945) (a mitochondrial ROS inhibitor), and the photon outputs were measured using a luminometer (Berthold Technologies, LUMAT LB 9507). An example of this assay is described for PLA and LLA tissue samples from normal and HF dogs in FIGS. 2A, B.

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- [0306] All patents, patent applications, patent application publications and other publications cited herein are hereby incorporated by reference as if set forth in their entirety.
- [0307] It should be understood that the methods, procedures, operations, composition, and systems illustrated in figures may be modified without departing from the spirit of the present disclosure. For example, these methods, procedures, operations, devices and systems may comprise more or fewer steps or components than appear herein, and these steps or components may be combined with one another, in part or in whole.
- [0308] Furthermore, the present disclosure is not to be limited in terms of the particular embodiments described in this application, which are intended as illustrations of various embodiments. Many modifications and variations can be made without departing from its scope and spirit. Functionally equivalent methods and apparatuses within the scope of the disclosure, in addition to those enumerated herein, will be apparent to those skilled in the art from the foregoing descriptions.

 SEQUENCE LISTING

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tatccatttc caagtcatag g

The invention claimed is:

1. A method of inhibiting oxidative stress in a subject having atrial or ventricular arrhythmias, ventricular failure or heart failure, comprising administering an effective amount of a NOX2 inhibitor agent to the subject, wherein said administering is under conditions such that a level of oxidative stress in myocardial tissue is reduced or eliminated.
2. The method of claim 1, wherein said arrhythmia comprises atrial fibrillation.
3. The method of claim 1, wherein the NOX2 shRNA is SEQ ID NO: 1.
4. The method of claim 1, wherein administering an effective amount of a NOX2 inhibitor agent to the subject comprises:
 - (a) providing an isolated therapeutic DNA comprising an small hairpin RNA (shRNA) against NOX2 mRNA (NOX2 shRNA) expression vector that encodes and expresses NOX2 shRNA in vivo; and
 - (b) administering the isolated therapeutic DNA to myocardial tissue of the subject.
5. The method of claim 4, wherein the myocardial tissue comprises at least one of atrial tissue or ventricle tissue.
6. The method of claim 4, wherein administering the isolated therapeutic DNA to myocardial tissue of the subject comprises injecting the isolated therapeutic DNA.
7. The method of claim 1, further comprising assessing a parameter of atrial tissue status in the subject.
8. The method of claim 7, wherein assessing a parameter of atrial tissue status in the subject comprises monitoring an electrophysiological measurement associated with AF or assessing fibrosis status for a region of the myocardial tissue after administering NOX2 inhibitor agent to the subject.
9. The method of claim 7, wherein assessing a parameter of atrial tissue status in the subject comprises monitoring an electrophysiological measurement associated with AF selected from AF onset, AF duration, conductivity and conductive inhomogeneity index.
10. A method of claim 2, further comprising assessing an AF characteristic following administering the isolated therapeutic DNA.
11. The method of claim 10, wherein the AF characteristic comprises at least one member selected from a group consisting of AF duration, AF episode inducibility, effective refractory periods and conduction inhomogeneity index.

12. The method of claim 1, further comprising assessing at least one member selected from a group consisting of NOX2 nucleic acid, NOX2 polypeptide, superoxide production and fibrosis.

13. A method of treating a subject having atrial or ventricular arrhythmias, ventricular failure or heart failure, comprising administering an effective amount of a NOX2 inhibitor agent to the subject, wherein said administering is under conditions such that a level of atrial or ventricular arrhythmias is reduced or eliminated.

14. The method of claim 13, wherein said arrhythmia comprises atrial fibrillation.

15. The method of claim 13, wherein administering an effective amount of a NOX2 inhibitor agent to the subject

- (a) providing an isolated therapeutic nucleic acid comprising a small hairpin RNA against NOX2 mRNA (NOX2 shRNA) in vivo; and
- (b) administering the isolated therapeutic nucleic acid to myocardial tissue of the subject.

16. The method of claim 15, further comprising: (c) assessing AF status for the myocardial tissue after administration of the therapeutic nucleic acid.

17. The method of claim 16, wherein the AF status comprises at least one member selected from the group consisting of AF duration, AF episode inducibility, effective refractory periods and conduction inhomogeneity index.

18. The method of claim 13, further comprising assessing at least one member selected from a group consisting of NOX2 nucleic acid, NOX2 polypeptide, superoxide production and fibrosis.

19. The method of claim 13, wherein the isolated therapeutic nucleic acid is expressed from an expression vector encoding the NOX2 shRNA.

20. A pharmaceutical composition for inhibiting oxidative stress in a subject having atrial or ventricular arrhythmias, ventricular failure or heart failure, comprising an isolated nucleic acid encoding a small hairpin RNA against NOX2 mRNA (NOX2 rhRNA).

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