The development and characterisation of a novel class of antiarrhythmic prodrugs

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The development and characterisation of a novel class of antiarrhythmic prodrugs

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Dedications

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Publications and Prizes

Papers

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Abstracts

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Abstract

Sudden cardiac death, caused by the lethal arrhythmia ventricular fibrillation during acute myocardial ischaemia and infarction, is a leading cause of death worldwide. Currently available antiarrhythmic drugs have failed to provide comprehensive protection against sudden cardiac death because the benefit afforded by these drugs is offset by systemic adverse effects, resulting in an unacceptably narrow therapeutic index. Consequently, they are used only sparingly in those at highest risk of sudden cardiac death and are not considered safe and effective enough for use in larger lower-risk populations, and thus a novel antiarrhythmic drug with a large therapeutic index is required.

The purpose of this research was to examine lidocaine N-oxide, a novel putative ischaemia-activated antiarrhythmic prodrug. The project aimed to test the hypothesis that lidocaine N-oxide is pharmacologically inactive prior to its hypoxia-activated conversion to lidocaine during myocardial ischaemia, affording the drug antiarrhythmic effectiveness in the absence of adverse effects. In *ex vivo* and *in vivo* rat models of acute myocardial ischaemia, it was shown that lidocaine N-oxide exhibited antiarrhythmic effectiveness without adverse effects, generating a translational therapeutic index far greater than that of lidocaine. Using a range of different techniques in the rat isolated heart, lidocaine N-oxide was shown to exhibit a portfolio of effects on arrhythmias and conduction that was identical to that of lidocaine and attributable to I_{Na} block, differing only in terms of lidocaine N-oxide's absolute dependency on the presence of ischaemia to exert an effect. In the rat isolated heart model, using a newly developed UHPLC-MS/MS analysis method, it was shown that lidocaine N-oxide was reduced to lidocaine selectively in the ischaemic myocardium.

The results generated in this thesis justify further preclinical and clinical development of lidocaine N-oxide as an ischaemia-activated antiarrhythmic prodrug for the prevention of sudden cardiac death.

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Abbreviations

A	Activated channel state
ADP	Adenosine diphosphate
ADR	Adverse drug reaction
AMI	Acute myocardial infarction
ANOVA	Analysis of variance
AP	Action potential
APD	Action potential duration
AQ4	Anakylaminoanthraquinone
AQ4N	Anakylaminoanthraquinone N-oxide
ATP	Adenosine triphosphate
AV	Atrioventricular
beats/min	Beats per minute
BG	Bigeminy
Ca^{2+}	Calcium ion
CAST	Cardiac Arrhythmia Suppression Trial
CHD	Coronary heart disease
Cl^-	Chloride ion
CNS	Central nervous system
CO_2	Carbon dioxide
CP	Creatine phosphate
CYP450	Cytochrome P450
°C	Degrees Celsius
DAD	Delayed afterdepolarisation
EAD	Early afterdepolarisation
ECG	Electrocardiogram
ERP	Effective refractory period
FFR	Force-frequency relationship
g	Gram
GI	Gastrointestinal
H^+	Hydrogen ion
H_2O	Water
H_2O_2	Hydrogen peroxide
HAP	Hypoxia-activated prodrug
HH	Hodgkin-Huxley
Hz	Hertz
I	Inactivated channel state

Abbreviations

i.p.	Intraperitoneal
i.v.	Intravenous
I_{CaL}	L-type Ca^{2+} current
ICD	Implantable cardioverter defibrillator
I_f	Cardiac hyperpolarisation-activated pacemaker current
I_k	Delayed rectifying K^+ current
I_{k1}	Inwardly rectifying current
I_{kr}	Rapidly activating component of the rectifying K^+ current
I_{ks}	Slowly activating component of the rectifying K^+ current
I_{Na}	Fast inward Na^+ current
IS	Internal standard
I_{to}	Transient outward current
IU	International unit
IVB	Intraventricular balloon
IZ	Ischaemic zone
K^+	Potassium ion
K_{ATP}	ATP-dependent K^+ channel
kg	Kilogram
k_I, k_A, k_R	Association rate to channel states
LAD	Left anterior descending (coronary artery)
LDH	Lactate dehydrogenase
l_I, l_A, l_R	Dissociation rate to channel states
LNO	Lidocaine N-oxide
LV	Left ventricle
LVEF	Left ventricular ejection fraction
mA	Milliamp
MEGX	Monoethylglycinexylidide
mg	Milligram
MI	Myocardial infarction
min	Minute
ml	Millilitre
mM	Millimoles per litre
mmHg	Millimetres of mercury
MRH	Modulated receptor hypothesis
MRM	Multiple reaction monitoring
ms	Millisecond
mV	Millivolt
m/z	Mass-to-charge ratio
MΩ	Megaohm
Na^+	Sodium ion

Abbreviations

NADPH	Nicotinamide adenine dinucleotide phosphate
ng	Nanogram
nm	Nanometre
NMR	Nuclear magnetic resonance
NSVF	Non-sustained ventricular fibrillation
O_2	Oxygen
PCI	Percutaneous coronary intervention
pH	The negative logarithm of the hydrogen ion concentration
P_i	Inorganic phosphate
PR	PR interval
PV	Pressure-volume
QT	QT interval
QT_{90}	QT interval measured at 90% repolarisation
R	Rested channel state
r^2	Coefficient of determination
RyR	Ryanodine receptor
S	Salvos
s	Second
SA	Sinoatrial
SC	Starling curve
SCA	Sudden cardiac arrest
SCD	Sudden cardiac death
SEM	Standard error of the mean
SERCA	Sarco/endoplasmic reticulum ATPase
SR	Sarcoplasmic reticulum
SVF	Sustained ventricular fibrillation
TDP	Torsade de pointes
TI	Therapeutic index
TTI	Translational therapeutic index
TVW	Total ventricular weight
UHPLC-MS/MS	Ultra-high performance liquid chromatography with tandem mass spectrometry
VF	Ventricular fibrillation
V_{max}	Upstroke velocity of the action potential
VPB	Ventricular premature beat
VT	Ventricular tachycardia
µg	Microgram
µl	Microlitre
µM	Micromoles per litre

Chapter 1: Introduction

1.1 Sudden cardiac death

Sudden cardiac death (SCD), defined as sudden death from cardiac causes, heralded by the abrupt loss of consciousness within one hour from the onset of symptoms (Priori et al., 2015), is responsible for 15-20% of all deaths in Western societies (Wong et al., 2019; Hayashi, Shimizu and Albert, 2015). It accounts for half of all cardiovascular disease fatalities (Myerburg and Junttila, 2012; Turakhia and Tseng, 2007; Adabag et al., 2010; Wong et al., 2019) which, itself, is the leading cause of death worldwide (World Health Organisation, 2018). The rate of SCD varies geographically, ranging between 40-100 per 100,000 in the general population (Hayashi, Shimizu and Albert, 2015; John et al., 2012; Wong et al., 2019), although this has decreased incrementally over time (Fox et al., 2004; Feng et al., 2017).

Approximately 80% of cases of SCD can be attributed to coronary heart disease (CHD), with the remaining cases triggered by hypertrophic or dilated cardiomyopathies and, less commonly, congenital heart conditions and inherited electrical abnormalities (Myerburg et al., 1997; Zipes and Wellens, 1998; Chugh et al., 2008; Myerburg and Junttila, 2012; Adabag et al., 2010). As a result, the trends concerning the prevalence of CHD and SCD are closely aligned. As the incidence of CHD rises with increasing age, the rate of SCD follows the same age-related trend and is highest in the older adult population (Chugh et al., 2004; Hayashi, Shimizu and Albert, 2015; Wong et al., 2019). Additionally, the employment of improved preventative measures has led to a decrease in the abundance, severity and fatality of CHD in recent decades (Luepker, 2008; Ford et al., 2007; Hellermann et al., 2002), resulting in a lower incidence of SCD in the general population (Fox et al., 2004; Feng et al., 2017).

However, the incidence of SCD has decreased at a slower rate than that of CHD incidence and mortality (Fox et al., 2004; Feng et al., 2017). Given this, and its remaining high prevalence worldwide, preventing SCD in CHD remains a major unmet clinical need.

1.1.1 Acute myocardial infarction

CHD is identified by characteristic lesions, known as atherosclerotic plaques, which, according to autopsy findings, can be detected in the arterial walls of up to 80% of SCD victims (Virmani, Burke and Farb, 2001). With CHD as an underlying condition, SCD is triggered most frequently by acute myocardial ischaemia (section 1.2) caused by atherosclerotic plaque rupture resulting in acute thrombosis (Davies, 1992; Koplan and Stevenson, 2009; Podrid and Myerburg, 2005). Plaque rupture followed by sustained myocardial ischaemia (>30 min) results in myocardial cell death (i.e. infarction), with this and acute myocardial ischaemia constituting the clinical syndrome known as acute myocardial infarction (AMI) (Thygesen, Alpert and White, 2007). When acute myocardial ischaemia occurs, the arrhythmias ventricular tachycardia (VT) and ventricular fibrillation (VF) are the primary triggers of sudden cardiac arrest (SCA) leading to SCD, which occurs in 90% of SCA cases occurring outside of the hospital (section 1.2) (Bayés de Luna, Coumel and Leclercq, 1989; Kauppila et al., 2018; Berdowski et al., 2010; Smolina, Wright and Rayner, 2012). The number of sudden deaths resulting from VF has declined in recent decades (Cobb et al., 2002) in proportion to the decline in prevalence of CHD (Fox et al., 2004; Feng et al., 2017). However, this decrease appeared to stabilise some years ago (Väyrynen et al., 2011), and VF now remains the primary cause of SCD during acute myocardial ischaemia and infarction (Kauppila et al., 2018).

1.1.1.1 Emergency treatment of AMI

Emergency care during suspected AMI due to myocardial ischaemia requires rapid diagnosis and treatment. Electrocardiogram (ECG) monitoring is used to determine whether rapid defibrillation of a ‘shockable’ arrhythmia, such as VF, is required to revert the heart back to sinus rhythm (Ibanez et al., 2018). Administration of morphine may be used to provide relief from pain and reduce the risk of pain-induced sympathetic activation (Ibanez et al., 2018). Rapid transport to a hospital is undertaken, after which treatments for preventing re-occurrence commence (Ibanez et al., 2018). If ischaemia is suspected (i.e. ST-segment elevation witnessed on the ECG (Thygesen et al., 2012)), swift reperfusion therapy involving either percutaneous coronary intervention (PCI), using a balloon or stent (Keeley, Boura and

Grines, 2003), or fibrinolytic drug administration (FTT Collaborative Group, 1994) is implemented (Ibanez et al., 2018). However, despite these emergency measures, up to two thirds of AMI fatalities occur shortly after symptom onset before the sufferer receives medical attention or reaches a hospital (Chambless et al., 1997; Koopman et al., 2013).

1.1.1.2 Preventing SCD following AMI

Within the first 30 days following AMI, one third of cases are fatal (Smolina, Wright and Rayner, 2012; Koopman et al., 2013). Following this period, AMI survivors are amongst those at highest risk of SCD (Myerburg et al., 1998; Huikuri, Castellanos and Myerburg, 2002), accounting for 36% of cardiac-related deaths during the first 3 years post-MI (Jokhadar et al., 2004), the basis of which may be heart failure and infarct-related arrhythmias or arrhythmias arising due to recurrent ischaemic events (Jokhadar et al., 2004; Adabag et al., 2008). Thus, device-based and pharmacological preventative strategies are employed to reduce the risk of death (Ibanez et al., 2018). The implantable cardioverter defibrillator (ICD) is a device used in SCD prevention, and patients with CHD, especially those post-MI, make up most recipients (Epstein et al., 2008, 2013). Supported by the results of clinical trials (Moss et al., 1996, 2002; Bardy et al., 2005), guidance recommends their use in patients with a compromised left ventricular (LV) ejection fraction (LVEF) (<40%) when assessed at least 40 days after AMI (Epstein et al., 2008, 2013), since early ICD implantation appears nonbeneficial (Steinbeck et al., 2009; Hohnloser et al., 2004). Early administration of antiarrhythmic pharmacotherapy is also not recommended since it can be harmful or ineffective (for reasons elaborated upon later) (Piccini et al., 2011; Priori et al., 2015). Thus, in the short-term period following AMI, during which the risk of post-MI SCD is highest (Pouleur et al., 2010; Adabag et al., 2008), neither device-based nor pharmacological antiarrhythmic therapy is indicated owing to the inadequate effectiveness or safety of current interventions.

Ancillary therapies are administered after the acute event and continued in the long term (after the first 30 days) with view to reducing mortality (Ibanez et al., 2018; Dan et al., 2018; Priori et al., 2015; Al-Khatib et al., 2018). These may include treatment with statins (Stenestrand and Wallentin, 2001) and anticoagulant and antiplatelet agents (Rothberg et al.,

2005) which help to prevent AMI reoccurrence and improve survival, albeit these agents self-evidently do not target the arrhythmias ultimately responsible for post-MI fatality. Routine β-blockade therapy is known to reduce post-MI mortality (Freemantle et al., 1999; β-Blocker Heart Attack Study Group, 1981; The Norwegian Multicenter Study Group, 1981), making these drugs a first line choice for preventing SCD in this population (Priori et al., 2015; Ibanez et al., 2018; Al-Khatib et al., 2018). However, despite their wide-spread use, VF leading to SCD is still prevalent post-MI (Myerburg et al., 1998; Huikuri, Castellanos and Myerburg, 2002; Jokhadar et al., 2004), thus the effectiveness of β-blockers as *antiarrhythmic* drugs in this context is questionable (section 1.3.2.1). Furthermore, whilst the ICD is undoubtably beneficial at terminating arrhythmias and reducing post-MI mortality (Moss et al., 2002, 1996; Bardy et al., 2005; Epstein et al., 2008, 2013), its use may be associated with psychological issues in anticipation of shock therapy (Sears and Conti, 2002), and the occurrence of inappropriate ICD shocks may reduce its mortality benefit by negatively impacting cardiac function (Van Rees et al., 2011; Daubert et al., 2008). Supplemental β-blockers are often prescribed to reduce inappropriate shocks (Priori et al., 2015).

Pharmacological antiarrhythmic therapy has been prescribed post-MI (Ibanez et al., 2018; Dan et al., 2018; Priori et al., 2015; Al-Khatib et al., 2018), but its use became restricted (Avanzini et al., 1995) following the negative results of two major clinical trials (Waldo et al., 1996; CAST Investigators, 1989). Consequently, current guidance actively dissuades the use of class 1a and 1c antiarrhythmic drugs due to an unfavourable balance between risk and benefit (Priori et al., 2015; Dan et al., 2018; Al-Khatib et al., 2018), the result of paradoxical proarrhythmia (i.e. the generation of new, or exacerbation of existing, arrhythmias) and other adverse drug reactions (ADRs) at therapeutic doses, mediated in the heart or elsewhere in the body, that lead to dose reduction or treatment withdrawal (Rutledge, Harris and Amsterdam, 1985; CAST Investigators, 1989; Morganroth and Goin, 1991; Teo, Yusuf and Furberg, 1993). Class 1b antiarrhythmics lidocaine and mexiletine represent a conditional exception to the move away from antiarrhythmic drugs, with their use permitted in tandem with ICD implantation (Abboud and Ehrlich, 2016) and when an individual's risk of ventricular arrhythmias is very high (Dan et al., 2018; Joint Formulary Committee, 2019; Al-Khatib et al., 2018), although lidocaine must be administered intravenous (i.v.) and so can't be used outside

the hospital setting. The class 3 antiarrhythmic amiodarone may also be used in this context (Dan et al., 2018; Priori et al., 2015; Al-Khatib et al., 2018), albeit ADRs are common and serious (Priori et al., 2015; Goldschlager et al., 2007).

1.1.2 Reducing the overall rate of SCD

Approximately half of sudden deaths from CHD occur when the underlying condition is undiagnosed, with death representing the first clinical manifestation of the disease (Kannel, Cupples and D'Agostino, 1987; Norris, 1998; Wong et al., 2019; John et al., 2012; Myerburg and Junttila, 2012). Therefore, because there are no truly safe and strongly effective antiarrhythmics that can be used in long term prophylaxis, the challenge is to identify those at greatest risk of SCD by risk-stratification methods, and focus on risk mitigation (rather than antiarrhythmic prophylaxis). In the general population, individual SCD risk prediction scores, such as SCORE or Framingham, are calculated based on non-modifiable risk factors such as age and gender, plus modifiable risk factors including smoking, blood pressure, total and LDL cholesterol levels, body mass index, and diabetes (Wellens et al., 2014). In those with known cardiovascular conditions, such as LV dysfunction and a previous MI, techniques such as cardiac imaging and ECG analysis can identify anatomical, autonomic and electrophysiological abnormalities that may help predict SCD in this high-risk population (Wellens et al., 2014; Deyell, Krahn and Goldberger, 2015; Arsenos et al., 2013), although LVEF is the most used predicator of SCD risk (Priori et al., 2015). Quantification of an individual's SCD risk can identify those that would benefit most from preventative strategies. However, before CHD is identified or AMI has occurred, few measures are available for those identified as at 'some' risk (Wellens et al., 2014; Priori et al., 2015), with current primary prevention measures acting to reduce cholesterol and blood pressure by statin and antihypertensive use, respectively, and implementing appropriate lifestyle changes (Gemmell et al., 2006).

The risk of SCD is highest in those with a history of cardiovascular conditions (i.e. AMI, SCA and LV dysfunction). However, these patients contribute only a tiny fraction of the total number of SCD cases, with the highest contributing group being those considered lowest-risk (i.e. the general population) (Myerburg et al., 1998; Huikuri, Castellanos and Myerburg, 2002) (Figure 1.1), who display insufficient risk-factors to qualify for the use of currently available

preventative measures, since they carry a risk of ADRs and are unproven in terms of efficacy in lower risk populations (Wellens et al., 2014; Priori et al., 2015). β-blockers are a first line drug in the prevention of SCD (Priori et al., 2015; Al-Khatib et al., 2018) especially in those with compromised LVEF and hypertension (Kezerashvili, Marzo and De Leon, 2012). However, a meta-analysis of clinical trials found that their benefit occurs primarily in high-risk patients (i.e. old, previous MI, LV dysfunction) with little benefit to lower-risk persons (Kezerashvili, Marzo and De Leon, 2012).

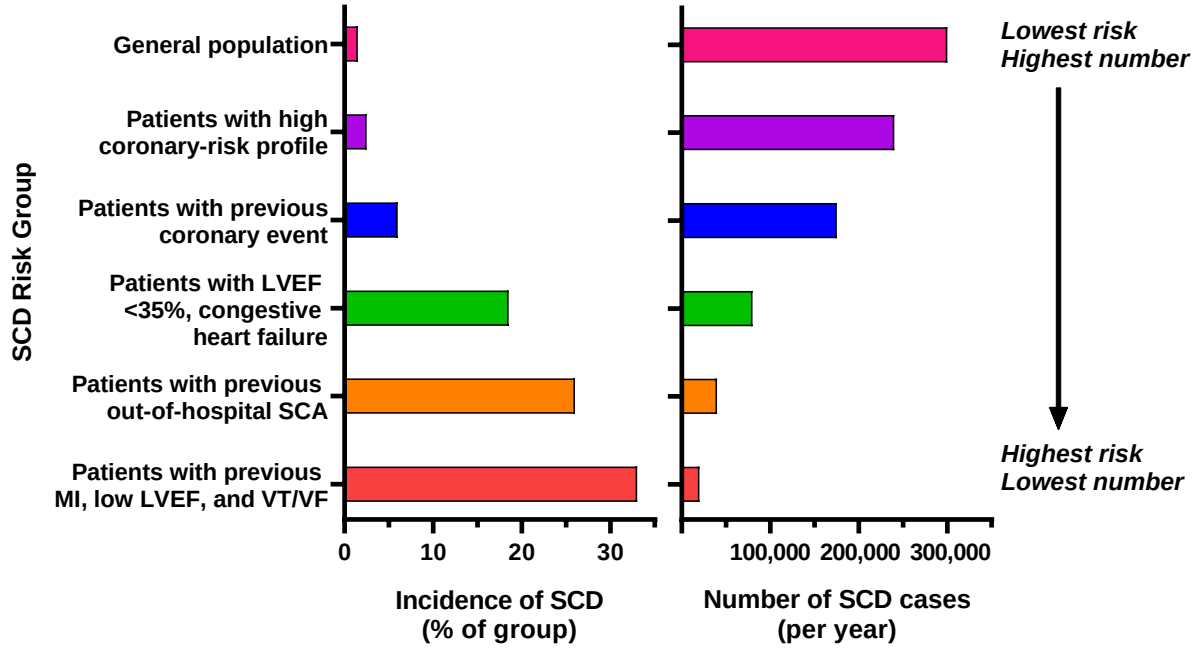


Figure 1.1 The incidence of SCD (% of group) and number of SCD cases (per year) in varying SCD risk groups, displaying the relevant contributions of each SCD risk group to the overall rate of SCD. Figure adapted from Myerburg et al. (1998) and Huikuri et al. (2002).

As noted above, mexiletine and amiodarone are the only antiarrhythmic drugs prescribed outside the hospital setting for prophylactic treatment of life-threatening ventricular arrhythmias that may result in SCD (Priori et al., 2015; Dan et al., 2018; Joint Formulary Committee, 2019; Goldschlager et al., 2007; Al-Khatib et al., 2018). However, these drugs are only used sparingly in those at highest risk of SCD and are not considered to be safe and effective enough for use in larger lower-risk populations (Priori et al., 2015; Joint Formulary Committee, 2019; Dan et al., 2018; Al-Khatib et al., 2018). For an antiarrhythmic drug to be

administered, the benefits of the drug's use must outweigh the risks. In cohorts at high-risk of SCD, the risk/benefit assessment is more likely to favour the use of an antiarrhythmic drug to obtain imminent vital antiarrhythmic benefit, but this is still dependent on the perceived risks and the tolerability of any ADRs. Any new drug developed for widespread use in the larger cohort of patients with lower SCD risk-profiles will need to be essentially devoid of ADRs. Currently, the benefit of available antiarrhythmic drug use is small, and the perceived risk of ADRs precludes their widespread use.

1.1.2.1 The need for a novel antiarrhythmic drug

In view of the above, a novel antiarrhythmic drug is required which displays a large therapeutic index (TI), defined as the ratio of the lowest dose of the drug which induces ADRs, to the lowest dose that leads to the desired pharmacological effect (i.e. prevention of ischaemia-induced VF and SCD) (Schneiderman et al., 1964). Developing a safe and effective drug requires an understanding of the mechanisms underpinning ischaemia-induced arrhythmogenesis, which may reveal targets for arrhythmia suppression (section 1.2). This may be facilitated by considering the basis for the failed strategies employed in previous attempts at developing antiarrhythmic drugs for this purpose (section 1.3).

1.2 Ischaemia-induced arrhythmogenesis

The electrophysiological changes that occur during acute myocardial ischaemia contribute to the initiation of lethal ischaemia-induced arrhythmias (Janse et al., 1980), most notably VT and VF (Kauppila et al., 2018). Acute myocardial ischaemia is defined, for the purpose of this thesis, as the reversible state of insufficient coronary arterial blood flow and inadequate perfusion and supply of oxygen and metabolic substrates to the myocardium, resulting in the inability to maintain normal function of the myocardium (relative to 'pre-ischaemia' cardiac function) (Hearse, 1994). The Lambeth Conventions defines VF as a sequence of consecutive ventricular complexes, with no diastolic pauses, that vary non-progressively with respect to peak-peak interval, height and intrinsic shape (Curtis et al., 2013). Arrhythmias arising as a result of ischaemia are categorised into two distinct phases (Curtis, 1998). Phase 1

arrhythmias occur most commonly during the first 30 min of ischaemia. During this period, the effects of ischaemia are reversible as the ischaemic tissue is still viable. Phase 2 arrhythmias arise later, approximately 1.5-2 hours after the onset of ischaemia, at which point tissue is becoming necrotic and a permanent infarct develops, resulting in irreversible injury (Harris, 1950; Opitz et al., 1995; Jennings and Ganote, 1974). The present thesis will primarily focus on phase 1 arrhythmias.

1.2.1 Potentially arrhythmogenic metabolic, ionic and electrophysiological changes during acute myocardial ischaemia

The myocardial tissue distal to a coronary occlusion site becomes deprived of oxygen-rich blood, triggering a cascade of metabolic, ionic and electrophysiological changes in the myocardium that may result in arrhythmogenic consequences including a flow of injury current and re-entry, as explained below.

1.2.1.1 Metabolic and ionic changes in response to acute ischaemia

Oxygen-deprivation in the ischaemic zone (IZ) triggers a substantial and rapid decrease in the intracellular levels of high energy phosphates, including adenosine triphosphate (ATP) and creatine phosphate (CP), and the intracellular accumulation of inorganic phosphate (P_i) and adenosine diphosphate (ADP) (Jones et al., 1976; Clarke, O'Connor and Willis, 1987; Steenbergen et al., 1987; Allen and Orchard, 1987). When oxygen trapped in the tissue distal to the occlusion begins to be consumed, energy metabolism in the IZ is quickly shifted from aerobic mitochondrial metabolism to anaerobic glycolysis (Jennings et al., 1990; Kloner and Jennings, 2001; Stanley, 2001; Stanley et al., 1997). The lack of ATP during ischaemia leads to disinhibition of the ATP-dependent K^+ (K_{ATP}) channel, allowing K^+ to move and accumulate extracellularly (Kléber, 1984; Gross and Auchampach, 1992), and the inhibition of ATP-dependent Na^+/K^+ ATPase, which under normal conditions facilitates the movement of $2K^+$ into the cell and $3Na^+$ out (Terkildsen, Crampin and Smith, 2007). Therefore, extracellular K^+ accumulation is substantial (from approximately 4 to >10 mM) within 10 min of ischaemia onset (Kléber, 1984) (Figure 1.2).

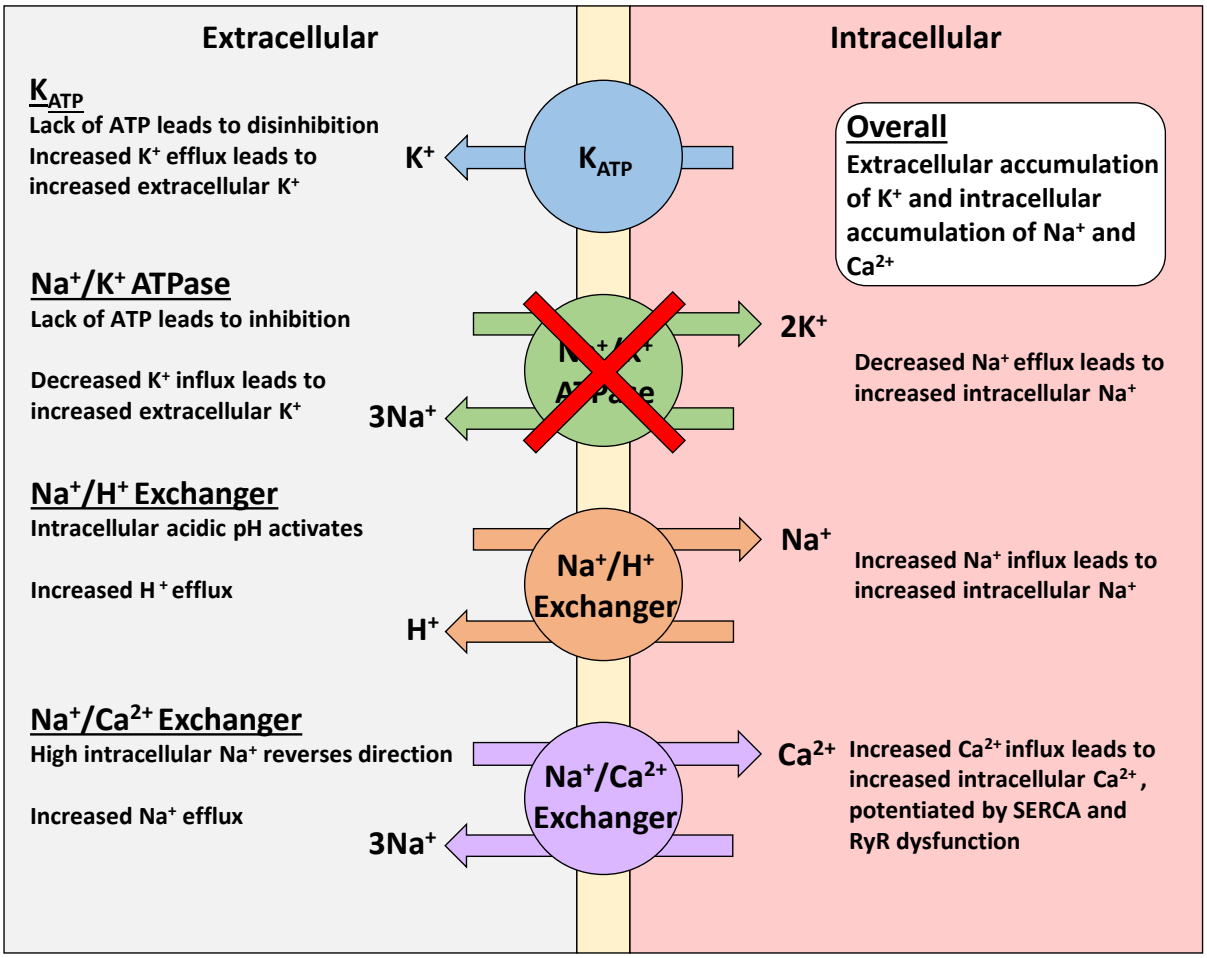


Figure 1.2 Diagram of the changes in ion homeostasis in response to acute myocardial ischaemia, resulting in the extracellular accumulation of K^+, and the intracellular accumulation of both Na^+ and Ca^{2+}.

In a normal myocyte, the diastolic membrane potential is approximately -90 mV. Within min of the onset of acute ischaemia, the diastolic membrane potential shifts to a more positive value near the K^+ equilibrium potential, approximately -60 mV, in parallel to changes in extracellular K^+ (Janse and Kléber, 1981) (Figure 1.3). This diastolic depolarisation alters the balance of the Na^+/Ca^{2+} exchanger to encourage Ca^{2+} loading, favouring the direction of ion transport to Ca^{2+} entry and $3Na^+$ export, elevating cytosolic Ca^{2+} (Chen and Li, 2012; Avkiran, 1999) (Figure 1.2). Furthermore, as by-products of anaerobic glycolysis, lactate, H^+ and CO_2 accumulate intracellularly during ischaemia causing the tissue pH to lower (Gettes et al., 1991). As a result, the Na^+/H^+ exchanger extrudes H^+, causing Na^+ to rise intracellularly (Figure 1.2). Consequently, the Na^+/Ca^{2+} exchanger attenuates the Na^+ accumulation in exchange for

an increase in intracellular Ca^{2+} (Avkiran, 1999) (Figure 1.2). Moreover, the lack of ATP and acidic pH during ischaemia further disrupts Ca^{2+} handling. The ability of the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) to return Ca^{2+} into the sarcoplasmic reticulum (SR) at the end of systole, to allow for relaxation during diastole, is limited (Stanley, 2001), and the amount of Ca^{2+} needed to maintain contractile function in the IZ at pre-ischaemic levels is increased (Fabiato and Fabiato, 1978). The increase in cytosolic Ca^{2+} early during ischaemia may also enhance the release of Ca^{2+} into the cytosol by the ryanodine receptor (RyR) by the mechanism of Ca^{2+}-induced Ca^{2+} release (Fauconnier et al., 2013). With Ca^{2+} handling disrupted and the demand for ATP not met during ischaemia, myocardial contraction suffers (Allen and Orchard, 1987; Stanley, 2001).

1.2.1.2 Electrophysiological changes in response to acute ischaemia

The hypoxia, acidosis and extracellular K^+ accumulation detailed above (section 1.2.1.1) are responsible for substantial changes to the morphology and propagation of the action potential (AP) (Figure 1.3). Depolarisation, the upstroke of the AP (phase 0), is caused by the fast inward sodium current (I_{Na}) generated by the opening of voltage-dependent Na^+ channels which quickly inactivate after opening (Carmeliet, 1999). In contrast to normal rapid AP generation, which sees quick and ubiquitous activation of fast Na^+ channels, the ischaemic AP has a reduced upstroke velocity (V_{max}) (Shaw and Rudy, 1997a) as a consequence of diastolic depolarisation leading to inactivation of a greater number of Na^+ channels at any given moment, reducing the proportion of Na^+ channels available for opening (Klabunde, 2017). This depolarisation-induced delayed recovery of I_{Na} and excitability also causes post-repolarisation refractoriness, whereby the refractory period extends beyond the point of complete repolarisation (Shaw and Rudy, 1997a) and slows conduction velocity (Kléber et al., 1986; Buchanan, Saito and Gettes, 1985) (Figure 1.3). In addition, a small persistent component of the cardiac Na^+ current, the late Na^+ current (I_{NaL}), present during the AP plateau, is increased in magnitude during ischaemia (Saint, 2006; Horváth et al., 2020). This impairs repolarisation and contributes to the rise in intracellular Na^+ concentration (Saint, 2006; Horváth et al., 2020). Lastly, the AP duration (APD) shortens, in part, owing to K_{ATP} channel disinhibition and early phase 3 repolarisation (Klabunde, 2017) (Figure 1.3).

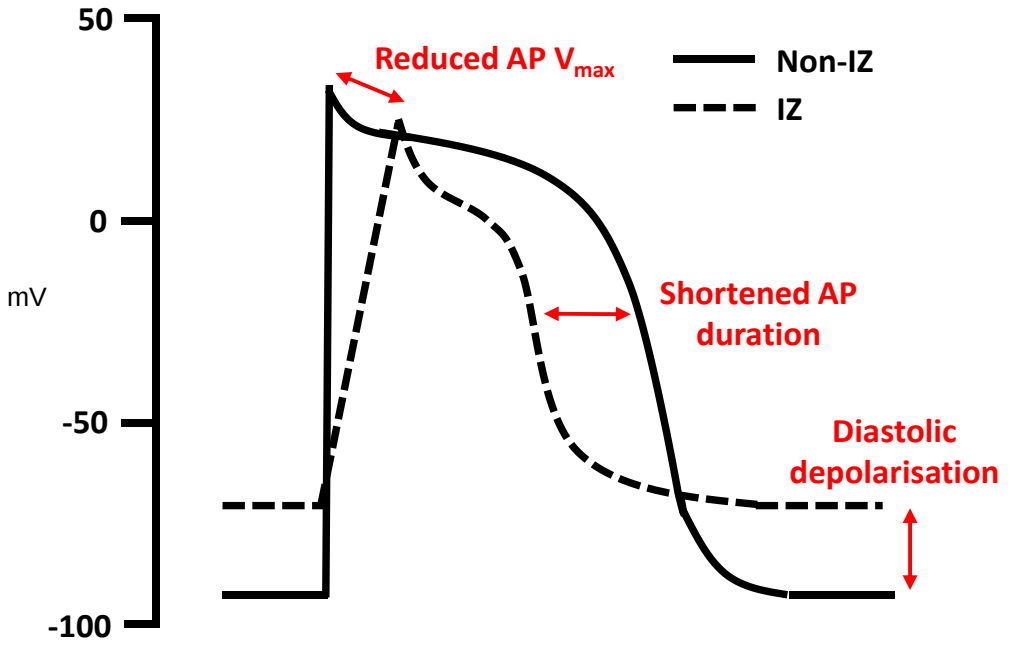


Figure 1.3 Diagram depicting the changes in ventricular action potential (AP) morphology in response to acute myocardial ischaemia, resulting in a reduced AP upstroke velocity (V_{max}), a shortened AP duration and diastolic membrane potential depolarisation in the IZ, in comparison to tissue outside the IZ (non-IZ). Figure adapted from Klabunde (2017).

As ischaemia progresses, an AP can still be generated via the L-type Ca^{2+} current (I_{CaL}), since the activation threshold of I_{CaL} is approximately -60 mV to -65 mV, and the threshold for inactivation is not reached until more sustained ischaemia obtains greater diastolic depolarisation (Shaw and Rudy, 1997a; Botting, Curtis and Walker, 1985; Cranefield, Wit and Hoffman, 1972; Reuter, 1974). Owing to the slower rate of charge transfer via I_{CaL}, the V_{max} of the AP is further reduced, causing slow conduction to worsen (Shaw and Rudy, 1997a; Botting, Curtis and Walker, 1985; Cranefield, Wit and Hoffman, 1972). Gap junctions, which maintain intercellular conductance and AP propagation, become increasingly uncoupled during ischaemia due to increased cytosolic Ca^{2+} and decreased intracellular pH, leading to a further reduction in conduction velocity (De Groot and Coronel, 2004; Kleber, 1992). Changes to AP morphology and conduction velocity create a border zone between the IZ and non-ischaemic tissue, wherein a significant voltage gradient exists, allowing the flow of injury current between the two regions (Janse et al., 1980; Janse and Kléber, 1981). This, and other

factors, contributes significantly to the initiation of ischaemia-induced arrhythmogenesis (section 1.2.2).

1.2.1.3 Infarct development

If ischaemia duration is less than 30 min, much of the tissue in the IZ can be salvaged if reperfused, depending primarily on the level of collateral flow which varies species-dependently (Schaper and Schaper, 1988; Maxwell, Hearse and Yellon, 1987). However, if ischaemia is prolonged, ischaemic tissue becomes infarcted and can trigger arrhythmias via mechanisms that may differ from those associated with early (non-infarcting) acute ischaemia (Harris, 1950; Opitz et al., 1998; Jennings and Ganote, 1974). Infarcted tissue becomes damaged and necrotic, and infarct development is associated with increases in intracellular lactate, cessation of anaerobic glycolysis (Jennings et al., 1990), a second phasic increase in extracellular K^+ accumulation (Hill and Gettes, 1980), as well as high intracellular concentrations of Na^+ and Ca^{2+} (Buja et al., 1975; Van Echteld et al., 1991). Myocardial cell death gives rise to clinical biomarkers of AMI. Increased circulating levels of creatine kinase, lactate dehydrogenase (LDH), and cardiac troponins, have been used to stratify suspected MI patients (Thygesen, Alpert and White, 2007), although cardiac troponin detection assays are now preferential (Thygesen et al., 2012). As cell death proliferates, intercellular conductance and gap junction association are progressively impaired (McCallister, Trapukdi and Neely, 1979), and conduction velocity will slow to the point of conduction block and electrical quiescence (Shaw and Rudy, 1997b; Hondeghem and Cotner, 1978). Following MI, LV remodelling and dysfunction may develop as a result and, due to the chronicity of CHD, subsequent episodes of myocardial ischaemia may develop and worsen LV dysfunction over time (Gheorghiade and Bonow, 1998).

1.2.2 Mechanisms of arrhythmogenesis

The metabolic, ionic and electrophysiological changes during acute ischaemia and infarction, as described above, may initiate arrhythmogenesis through one (or a combination) of three mechanisms; re-entry, abnormal or enhanced automaticity and triggered activity (Gaztanaga, Marchlinski and Betensky, 2012; Antzelevitch and Burashnikov, 2011; Tse, 2016; Klabunde,

2017). As the electrophysiological milieu changes as ischaemia persists, the mechanisms underpinning arrhythmogenesis change. Phase 1 arrhythmogenesis, occurring during acute ischaemia, is primarily the result of the flow of injury current (section 1.2.1.2) and re-entry (Curtis and Hearse, 1989a; Janse and Kléber, 1981; Clements-Jewery, Hearse and Curtis, 2005). On the other hand, phase 2 arrhythmias associated with infarction and heart failure are most commonly the result of either abnormal or enhanced automaticity, triggered activity and re-entry (Coronel et al., 2013; Clements-Jewery, Hearse and Curtis, 2005).

1.2.2.1 Re-entry

Re-entry occurs when a propagating AP fails to terminate and reactivates a region of myocardium that has recovered from refractoriness (Janse et al., 1980), and may be responsible for arrhythmia initiation and maintenance during myocardial ischaemia and infarction (Janse and Wit, 1989). Re-entry occurring during acute ischaemia is thought to initiate from ectopic activity facilitated by the flow of injury current between the IZ and non-ischaemic myocardium (Janse et al., 1980, 1986; Coronel et al., 1991) down a voltage gradient set up by differences in conduction velocity and AP morphology between the two regions (Janse et al., 1980; Janse and Kléber, 1981), leading to the re-excitation of normal cells or those in the ischaemic border zone (Janse et al., 1980; Coronel et al., 1991). This voltage gradient, and decreased intercellular conductance and excitability in the IZ, leads to local source-sink relationship mismatch, i.e. when the current provided by the excitation wavefront (the source) is insufficient to excite the tissue ahead (the sink) (Antzelevitch, 2001), which correlates to the development of unidirectional block (Romero et al., 2009). The pre-requisites for re-entry to initiate an arrhythmia, namely conduction delay and block, have been demonstrated in phase 1 arrhythmogenesis during acute myocardial ischaemia that follows ligation of the septal artery in anaesthetised dogs (El Sherif, Sherlag and Lazzara, 1975). Another study reproduced these findings in regionally ischaemic dog and pig hearts and revealed the important roles of both the subendocardium and the His-Purkinje system in the development of unidirectional block and re-entry resulting in VF during acute ischaemia (Janse et al., 1986). Given this, it is likely that acute subendocardial ischaemia causes diastolic depolarisation and inactivation of Na^+ channels, leading to the inability of Purkinje fibres to

deliver normal excitation to this myocardium (i.e. source-sink mismatch), and eventually an area of unidirectional block at the Purkinje fibre-muscle junction (Gilmour and Watanabe, 1994). Other, larger, conducting pathways originating from the Purkinje fibres provide a retrograde stimulus that conducts slowly through the subendocardial IZ towards its point of origin, establishing a re-entrant circuit (Gilmour and Watanabe, 1994). Whilst VT is characterised by the presence of one large re-entrant circuit, VF is the result of several smaller circuits with distinct paths (Janse et al., 1980).

Re-entry will persist when the following three criteria are met: (1) an area of unidirectional block exists in the conducting pathway, (2) the propagating wavefront progresses along a distinct pathway, uninterrupted, returning to its point of origin before re-entering the same conducting pathway, and (3) conduction through the pathway is sufficiently slowed in tissue proximal to the area of unidirectional block, producing an 'excitable gap' of myocardium between the 'head' of the propagating wavefront and the 'tail' of the wavefront, such that there is fully recovered tissue in the circuit that may be re-excited by the propagating wavefront, maintaining the re-entrant circuit (Antzelevitch and Burashnikov, 2011; Gaztanaga, Marchlinski and Betensky, 2012; Tse, 2016). The wavelength, defined as the pathway length occupied by the impulse and refractory to re-excitation, must be shorter, or equal to, the re-entrant circuit length (Antzelevitch and Burashnikov, 2011) and is determined by the following equation:

Wavelength = conduction velocity x refractory period

Therefore, a reduction in either the conduction velocity or APD (determinant of the refractory period) will shorten the re-entrant wavelength and encourage the maintenance of re-entry (Antzelevitch and Burashnikov, 2011).

1.2.2.2 Abnormal or Enhanced automaticity

Abnormal or enhanced automaticity is thought to underpin arrhythmias during infarct development, rather than acute ischaemia (Clements-Jewery, Hearse and Curtis, 2005; Janse and Wit, 1989). Enhanced automaticity may occur when the mechanism of overdrive

suppression fails (Tse, 2016). Overdrive suppression ensures that the higher rates of activation in the sinoatrial (SA) node, the heart's primary pacemaker (firing rate approx. 60-100 beats/min), overdrives the activation of latent subsidiary pacemakers in, for example, the atrioventricular (AV) node (firing rate approx. 40-60 beats/min) and the His-Purkinje system (firing rate approx. 20-40 beats/min) (Tse, 2016). Abnormal automaticity describes when non-pacemaker cells develop automaticity as a consequence of the diastolic membrane potential becoming depolarised to a value between -70 mV and -30 mV, particularly when extracellular K^+ concentration increases, resulting in repetitive and inappropriate impulse formation (Gaztanaga, Marchlinski and Betensky, 2012; Antzelevitch and Burashnikov, 2011).

1.2.2.3 Triggered activity

Triggered activity has been implicated as a cause of arrhythmias during infarction, heart failure and drug-induced proarrhythmia (Janse and Wit, 1989; Rubart and Zipes, 2005; Roden, 2004) and refers to impulse initiation as a result of afterdepolarisations, a term which describes membrane potential oscillations after an AP which, if they reach the threshold potential, cause the cells to depolarise and generate an AP. This AP may then act as the trigger for subsequent afterdepolarisations, from which a tachyarrhythmia can occur. Early afterdepolarisations (EADs) occur during repolarisation and delayed afterdepolarisations (DADs) occur shortly after repolarisation (Gaztanaga, Marchlinski and Betensky, 2012; Antzelevitch and Burashnikov, 2011; Tse, 2016).

EADs may occur when the APD is prolonged, when there is a net inward current which may overcome or reverse repolarisation, as a result of pathology or drug-induced reduced outward current (K^+) or increased inward current (Ca^{2+}) (Weiss et al., 2010). Torsades de pointes (TDP), a potentially lethal ventricular arrhythmia, and arrhythmias associated with inherited long-QT syndrome, ventricular hypertrophy and heart failure can result from EADs (Roden, 2004). DADs, however, result from intracellular diastolic Ca^{2+} overload, resulting in Ca^{2+} mediated membrane oscillations. Sufficiently high intracellular Ca^{2+} after repolarisation, but before subsequent depolarisation, will affect Na^+/Ca^{2+} exchange and lead to Na^+ influx, culminating in inappropriate AP generation (Tse, 2016). The increased intracellular Ca^{2+} is

caused by 'leaky' RyRs which allow Ca^{2+} to leak from the SR into the cytosol during diastole (Rubart and Zipes, 2005; Fink, Noble and Noble, 2011). Abnormalities in RyR function have been detected in heart failure, the pathological condition most associated with DADs (Rubart and Zipes, 2005). In addition, the increase in intracellular Na^{+}, and consequent Ca^{2+} overload, resulting from increased I_{NaL} during ischaemia may contribute to the development of afterdepolarisations (Saint, 2006; Antzelevitch et al., 2014).

Therefore, an antiarrhythmic drug developed with the aim to prevent ischaemia-induced VF would need to prevent re-entry. However, to have the greatest impact on SCD, an antiarrhythmic drug would need to block all the above arrhythmogenic mechanisms that may lead to VF, since acute ischaemia transitions to infarction, recruiting multiple mechanisms of arrhythmogenesis due to differing arrhythmogenic stimuli.

1.3 Pharmacological strategies to prevent ischaemia-induced VF

The development of antiarrhythmic drugs to prevent ischaemia-induced VF has been fraught with disappointments in clinical trials. Flecainide and D-sotalol were billed as potential anti-VF agents, but instead were paradoxically proarrhythmic (CAST Investigators, 1989; Waldo et al., 1996; Clements-Jewery, Andrag and Curtis, 2009). Other antiarrhythmic drugs were ineffective in clinical trials and caused ADRs (Moss et al., 1988; The Danish Study Group on Verapamil in Myocardial Infarction, 1990). As noted above, the class 1b drug mexiletine and class 3 drug amiodarone are the only antiarrhythmics currently prescribed outside the hospital setting for life-threatening ventricular arrhythmias (Priori et al., 2015; Dan et al., 2018; Joint Formulary Committee, 2019; Al-Khatib et al., 2018). However, their use is associated with common and serious ADRs (Priori et al., 2015; Dan et al., 2018; Joint Formulary Committee, 2019; Goldschlager et al., 2007). To obtain a novel antiarrhythmic drug with an optimal TI it is important to ensure the risk of ADRs is low by, for example, increasing the drug's disease-selectivity (i.e. limiting the drug's pharmacological actions to tachyarrhythmias arising during acute ischaemia, and/or the ischaemic territory itself, thereby minimising undesired pharmacological effects). For the purpose of this thesis, the

term *selectivity* is used to describe a drug that preferentially binds to one receptor or target over another, whereas *specificity* refers to a drug's ability to act via a receptor or target to exert a desired effect. Given this, the term *ischaemia-selectivity* is used in this thesis to describe a drug that displays pharmacological activity preferentially under conditions of acute myocardial ischaemia, and the term *tachycardia-selectivity* is used to refer to a drugs ability to act preferentially at high frequency rhythms (e.g. VT and VF). The modulated receptor hypothesis (MRH) (Hondeghem and Katzung, 1977, 1984) explains how the pharmacological targeting of ion channels can theoretically confer these two properties in anti-VF drug development.

1.3.1 The modulated receptor hypothesis

The MRH describes three structural configurations that ion channels may assume: the rested (*R*) state, the activated (*A*) state and the inactivated (*I*) state, each with associated properties (Hondeghem and Katzung, 1984, 1977) (Figure 1.4). The probability that a Na^+ channel is in one of these states is voltage- and time-dependent. Na^+ channels have a threshold membrane potential of approximately -60 mV, at which channels will transition from the closed (but openable) *R* state into the open *A* state, permitting transmembrane movement of Na^+. Following a brief open period (a few ms), Na^+ channels transition into the closed (and inexcitable) *I* state and, after repolarisation and a period of refractoriness, channels transition back into the *R* state (Hondeghem and Katzung, 1977) (Figure 1.4). The transition between states is governed by two parts of the channel, the inactivation gate and the activation gate, which change in structure in response to changes in membrane potential, but with different sensitivities and rates of structural change. The Na^+ channel consists of α and β subunits. The α subunits are organised into 4 domains (DI-DIV) with 6 transmembrane segments (S1-S6). The S1-S4 segments are responsible for the channel's voltage-sensing properties and are coupled to the S5-S6 segments which form the channel's pore (Fozzard, Sheets and Hanck, 2011; Nguyen et al., 2019; Pless et al., 2011). The Na^+ channel's activation gate is located where the S6 segments meet at the cytoplasmic end of the channel's pore. Changes in membrane voltage during depolarisation are sensed by the S4 segments near the extracellular channel surface, causing them to move. Due to linkage between the S4 and S5-S6 segments,

this movement causes the S6 segments to hinge and open the channel pore (Fozzard, Sheets and Hanck, 2011). In contrast, the inactivation gating mechanism is mediated by a separate intracellular linker between DIII and DIV of the channel which, in response to changes in voltage sensed by the S4 segments, moves and occludes the channels pore, preventing Na^+ conductance (Fozzard, Sheets and Hanck, 2011; Nguyen et al., 2019). These conformational changes are reversed during repolarisation (Fozzard, Sheets and Hanck, 2011). For the channel to be open and ion permeable, both the voltage-sensitive inactivation and activation gates of the channel must be in the open configuration (Hodgkin and Huxley, 1952; Hondeghem and Katzung, 1984). In the heart, the *R* state is favoured by negative membrane potentials at diastole when channels are excitable. The *A* state is most prevalent during the AP upstroke rendering channels open allowing rapid Na^+ influx, and the *I* state predominates during the AP plateau (Hondeghem, 1987) (Figure 1.4).

Due to conformational changes in the ion channel, and consequently the structure and accessibility of the drug binding site within the channel's pore, during state transitions, antiarrhythmic drugs have different association (k_R, k_A and k_I) and dissociation (l_R, l_A and l_I) rate constants for each channel state, i.e. drug affinity for the ion channel receptor site varies state-dependently (Hondeghem, 1987; Hondeghem and Katzung, 1984, 1977) (Figure 1.4). The key point with respect to the actions of antiarrhythmic drugs is that drug-bound channels, in contrast to drug-free channels, are unable to conduct ions. Thus, simply put, antiarrhythmic drugs prevent conduction abnormalities by disallowing the ion movement that facilitates them. In addition, the voltage-dependence of drug-bound channels is shifted to more negative potentials, causing them to accumulate in the *I* state, particularly when tissue is depolarised (Hondeghem, 1987; Hondeghem and Katzung, 1984, 1977; Tamargo, Valenzuela and Delpon, 1989). Whilst high concentrations of antiarrhythmic drugs may block all channel states, clinically useful antiarrhythmic drugs in therapeutic concentrations (µM range) preferentially bind to the *I* and *A* states of the channel versus the *R* state, affording them voltage-dependent block and use-dependent block (Hondeghem, 1987; Hondeghem and Katzung, 1984).

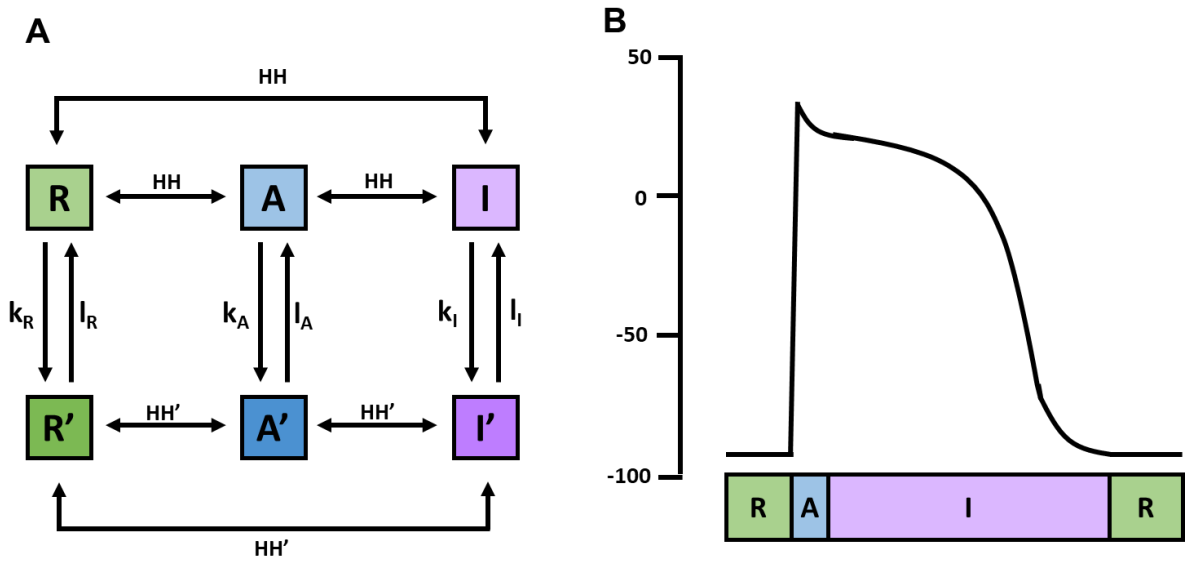


Figure 1.4 A) Diagram of the modulated receptor hypothesis for antiarrhythmic drug action and B) the relative time spent in each channel state during a ventriuclar action potential (AP). Na^+ channels exist in three states; rested (*R*), activated (*A*) and inactivated (*I*). Drug bound states (*R*', *A*', *I*') cannot conduct ions. Association (k_R, k_A and k_I) and dissociation (l_R, l_A and l_I) rate constants determine drug binding and unbinding to each channel state. State transitions are governed by Hodgkin and Huxley (HH) rate constants, modified by drug binding (HH'). Figure adapted from Hondeghem and Katzung (1984).

1.3.1.1 Voltage-dependent block

Na^+ channels transition from their *R* state to the *A* state and then the *I* state during the AP. Ischaemia-induced diastolic depolarisation (Janse and Kléber, 1981) increases the probability of Na^+ channels shifting to their *A* state and then subsequently accumulating in their *I* state (Hondeghem and Katzung, 1984). Consequently, antiarrhythmic drugs with greater affinity for the *I* state of the ion channel induce greater channel block in depolarised ischaemic tissue (Hondeghem and Katzung, 1984). Drug-induced channel block during ischaemia, in which conduction velocity is already slowed, will induce conduction block and termination of re-entrant pathways (Hondeghem and Katzung, 1984). Conversely, a drug with greatest affinity for the *R* state would cause greater channel block in polarised tissue, i.e. non-ischaemic tissue, which is likely to be proarrhythmic by slowing normal conduction and facilitating re-entry (Hondeghem and Katzung, 1984). Additionally, drug association is enhanced by depolarisation

and drug dissociation is slowed, therefore in ischaemic tissue the transition between the *I* state of drug-bound channels back to their drug-free *R* state is slowed and cumulative channel block occurs. Thus, voltage-dependent block affords antiarrhythmic drugs ischaemia-selectivity (Hondeghem and Katzung, 1984).

1.3.1.2 Use-dependent block

As a result of the greater affinity for the *I* and *A* channel states, and relative low affinity for the *R* state, possessed by antiarrhythmic drugs, drug-induced channel block is present during each AP (systole) and channels subsequently recover from this block during diastole. If the period of rest in between each AP is too short for a large proportion of channels to achieve full recovery from drug-induced block, then this will result in residual channel block at the start of the next AP (Hondeghem, 1987; Hondeghem and Katzung, 1984). This is the case during tachycardias, which generate more APs per unit time and, in comparison to slower heart rhythms, increase the amount of time spent in the *A* state and *I* state. The result is cumulative drug-induced channel block during tachyarrhythmias (e.g. VT and VF) versus that observed at sinus rhythm, affording antiarrhythmic drugs with tachycardia-selectivity (Hondeghem and Katzung, 1984).

1.3.2 Previous antiarrhythmic drugs developed to prevent SCD

Many attempts have made to develop antiarrhythmic drugs to prevent ischaemia-induced VF and reduce SCD, although thus far none of these approaches have been harnessed effectively to generate a safe *and* effective antiarrhythmic drug for widespread use. However, these attempts may be used to inform the development of novel antiarrhythmic drugs by interrogating their mechanisms of benefit and adversity.

1.3.2.1 β-blockers

As detailed in sections 1.1.1 and 1.1.2, β-blockers are used in the prevention of SCD. However, their benefit cannot be attributed exclusively to the prevention of ischaemia-induced VF. Whilst β-blockers do reduce arrhythmias following MI, they do not abolish them (Hjalmarson,

1997), and arrhythmogenic death is still common in this patient group (Myerburg et al., 1998; Huikuri, Castellanos and Myerburg, 2002; Jokhadar et al., 2004). Given that β-blockers may not benefit those without concomitant heart failure (Dondo et al., 2017), and are most beneficial in high risk comorbid populations (Kezerashvili, Marzo and De Leon, 2012), the benefit from β-blockers may instead be related to reducing the 'demand' on the heart by preventing β_1- and β_2-receptor-mediated sympathetic overstimulation of the myocardium, as well as reducing myocardial workload via blood pressure reduction (via vasodilation), slowing the heart rate (reducing cardiac output), and the long term improvement of haemodynamic factors such as LVEF (Kezerashvili, Marzo and De Leon, 2012; DiNicolantonio et al., 2013; Hjalmarson, 1997). Endogenous catecholamines, however, may provide an arrhythmogenic substrate during acute ischaemia, although the literature concerning this is inconsistent (Pugsley, Clements-Jewery and Curtis, 2003). The accumulation of endogenous catecholamines in relation to myocardial ischaemia has been detected *in vivo* (Schomig et al., 1984; Lameris et al., 2000), however it does not correlate to the timeline of phase 1 VF (Curtis, 1998). Additionally, since regionally ischaemic isolated (thus denervated) perfused hearts experience phase 1 VF reproducibly and predictably (Curtis, 1998), it is logical to conclude that, whilst catecholamines may facilitate ischaemia-induced VF in certain scenarios (Wilder et al., 2018), they are not essential for ischaemia-induced VF to occur. Therefore, whilst β-blockers are of benefit for reducing the risk of SCD in certain populations, their role as an anti-VF drugs is questionable.

1.3.2.2 Bradycardic agents

Bradycardic agents have also been considered for use in the context of CHD with view to reduce myocardial 'workload' without compromising blood pressure or LV contractile function (Koruth et al., 2017). Heart rate is controlled principally by SA node automaticity, depolarisation of which forms phase 4 of the AP and is governed by the cardiac hyperpolarisation-activated pacemaker current (I_f) (Baruscotti, Bucchi and DiFrancesco, 2005). Ivabradine is a selective I_f blocking drug which, in animal models of acute ischaemia (via coronary ligation), has been shown to reduce heart rate and consequently decrease the size of the hypoxic region (Vaillant et al., 2011) and the risk of ventricular arrhythmias

(Marciszek et al., 2020; Vaillant et al., 2011), without having a detrimental effect on LV contractile function (Vaillant et al., 2011). In the BEAUTIFUL trial, however, despite lowering heart rate ivabradine failed to reduce cardiovascular death or hospital admission due to MI or worsening heart failure in patients with stable CHD with LV dysfunction (Fox et al., 2008). Though, in a subgroup of patients whose baseline heart rate was >70 beats/min, ivabradine did reduce the risk of hospitalisation due to MI (Fox et al., 2008). But, in the SIGNIFY trial, which enrolled stable CHD patients without LV dysfunction, ivabradine treatment did not improve cardiovascular outcomes or reduce the rate of cardiovascular death, even when baseline heart rate was >70 beats/min (Fox et al., 2014). Therefore, ivabradine appears to improve cardiovascular outcomes only in a subset of CHD patients with concomitant heart failure and an elevated heart rate. In particular, the drug's lack of effect on cardiovascular death is an important factor to prompt consideration of other agents to prevent ischaemia-induced VF and SCD.

1.3.2.3 K^+ channel blockers

1.3.2.3.1 I_{kr} channel blockers

The delayed rectifier K^+ current (I_k), which is formed from a rapidly activating (I_{kr}) and a slow activating (I_{ks}) component (Sanguinetti and Jurkiewicz, 1990), mediates the repolarisation phase of the AP from its plateau, determining the refractory period and APD (Carmeliet and Mubagwa, 1998). Drugs that block I_{kr} delay repolarisation, prolong APD and extend the refractory period, reducing the probability of re-entrant circuits initiating by narrowing the 'excitable gap' (section 1.2.2.1). Antiarrhythmic drugs that block I_{kr} (e.g. D-sotalol and dofetilide) are termed class 3 drugs (Hondeghem and Snyders, 1990). In addition, class 1a drugs (e.g. quinidine), which block Na^+ channels, also block I_{kr} and prolong APD, and amiodarone, which displays non-selective pharmacology, blocking Na^+ and Ca^{2+} channels and displaying antiadrenergic actions in addition to prolonging APD by I_{kr} blockade, are considered to have class 3 effects (Hondeghem and Snyders, 1990).

In clinical trials, however, class 3 drugs have yielded disappointing results. D-sotalol increased arrhythmic death versus placebo post-MI causing its trial to be terminated early (Waldo et al.,

1996). Dofetilide also failed to improve mortality and caused TDP in 7 patients (Køber et al., 2000). Indeed, drugs which block I_{kr} prolong APD most profoundly at slower heart rates (Hondeghem and Snyders, 1990) due to the relative contributions of I_{kr} and I_{ks} to repolarisation differing according to heart rate (Sanguinetti and Jurkiewicz, 1990; Lu et al., 2001). Thus, selective I_{kr} blockers increase the risk of TDP, making them paradoxically proarrhythmic (Hondeghem and Snyders, 1990) and unsuitable for use in SCD prevention (Priori et al., 2015) or post-MI (Ibanez et al., 2018). Amiodarone is the most successful class 3 drug, still indicated for use in those at high-risk of ventricular arrhythmias (Priori et al., 2015; Dan et al., 2018; Goldschlager et al., 2007; Al-Khatib et al., 2018). It produces similar APD prolongation irrespective of heart rate (Hondeghem and Snyders, 1990) which, combined with its other pharmacological effects, means amiodarone use has a low risk of TDP (Hohnloser and Singh, 1995). In a clinical trial meta-analysis, chronic amiodarone use in heart failure or post-MI reduced overall mortality by 13% and arrhythmogenic death by 29% (Amiodarone Trials Meta-Analysis Investigators, 1997) although these findings have not been consistently reproduced (Boutitie et al., 1999; Piccini, Berger and O'Connor, 2009). In the acute setting, however, whilst amiodarone may prevent VF during out-of-hospital SCA, improving survival *en route* to hospitalisation (Kudenchuk et al., 1999), this benefit does not increase the likelihood of survival to hospital discharge (Kudenchuk et al., 2016). In addition, amiodarone causes serious ADRs at therapeutic doses (e.g. pulmonary fibrosis, thyroid problems, neuropathies, hepatotoxicity, bradycardia, photosensitivity and skin discolouration), with ADR risk rising with treatment duration and dose (Priori et al., 2015; Goldschlager et al., 2007). As a result, cumulative ADRs, compromising the drug's safety and narrowing its TI, preclude long-term use (Priori et al., 2015; Goldschlager et al., 2007).

1.3.2.3.2 K_{ATP} channel blockers

The movement of K^+ through the K_{ATP} channel is usually inhibited by the presence of ATP (Gross and Auchampach, 1992), however, during ischaemia ATP depletes and the K_{ATP} channel becomes disinhibited, allowing for rapid accumulation of K^+ into the extracellular space (section 1.2.1.1). The time course of change in extracellular K^+ concentration in the IZ coincides with the occurrence of ischaemia-induced VF, suggesting the former may contribute

to the latter (Curtis, 1991; Curtis and Hearse, 1989a). Blockade of K^+ efflux through K_{ATP} channels therefore has the potential to be antiarrhythmic by reducing extracellular K^+ accumulation in the IZ. Glibenclamide is a K_{ATP} channel blocker investigated as a potential anti-VF agent. However, rather than preventing VF, the drug has been shown to be defibrillatory in rat isolated hearts (reducing VF duration) (Bril, Laville and Gout, 1992; D'Alonzo et al., 1994; Rees and Curtis, 1995). Other studies have revealed glibenclamide's proarrhythmic tendencies (Bernauer, 1997) and propensity to induce bradycardia (Rees and Curtis, 1995; Bril, Laville and Gout, 1992) and reduce LV contractility (Bril, Laville and Gout, 1992; D'Alonzo et al., 1994). Additionally, the antifibrillatory effects of glibenclamide do not appear to result from the inhibition of ischaemic K^+ loss, and therefore may be due to another unidentified mechanism (Gwilt et al., 1992). This observation, and the fact that K_{ATP} channel openers may also be antiarrhythmic (Vegh et al., 1996), may explain why glibenclamide is not a suitable anti-VF agent.

1.3.2.4 Ca^{2+} channel blockers

Ca^{2+} handling is disturbed during acute myocardial ischaemia, causing cytosolic diastolic Ca^{2+} to become elevated (section 1.2.1.1). In addition, whilst I_{CaL} normally contributes to AP upstroke generation only in the SA node (Brown, 1982) and the plateau phase of the AP in atrial, Purkinje and ventricular tissue (Reuter, 1974), during ischaemia the contribution of I_{CaL} to the AP upstroke is increased as I_{Na} becomes progressively inactivated, thereby contributing to ischaemia-induced conduction slowing and APD shortening (section 1.2.1.2). When I_{Na} is inactivated owing to ischaemia, block of I_{CaL} may convert slowed conduction to bidirectional conduction block (Curtis, 1990).

Class 4 antiarrhythmics (e.g. verapamil and diltiazem) block I_{CaL} relatively selectively in the L-type Ca^{2+} channel's *I* state (Lee and Tsien, 1983; Kanaya, 1983) and therefore exhibit a degree of use-dependent and voltage-dependent block (Hondeghem and Katzung, 1984). However, by virtue of their voltage-dependence, I_{CaL} blockers act preferentially in the vasculature, since the membrane potential of smooth muscle cells is maintained at more positive voltages (~30-50 mV) (Steedman, 1966). Therefore, there is a higher probability that L-type Ca^{2+} channels

will be in their *I* state in the vasculature and, as a result, all class 4 agents have a greater selectivity for the vasculature over the myocardium (Sun and Triggle, 1995). In the heart, diltiazem and verapamil act preferentially in the nodal regions compared with the ventricular myocardium due to their voltage-dependence (Narimatsu and Taira, 1976). I_{CaL} blockers, particularly verapamil and diltiazem, have demonstrated antiarrhythmic effectiveness against ischaemia-induced VF in *ex vivo* and *in vivo* animal models, but these effects are confounded by the dysregulation of other Ca^{2+}-mediated aspects of physiology resulting from their relative selectivity for the vasculature and nodal regions (Curtis and Walker, 1986; Curtis, MacLeod and Walker, 1984; Thandroyen, 1982; Peter et al., 1983; Mertz and Kaplan, 1982; Rowland et al., 1983; Satoh, Yanagisawa and Taira, 1980; Farkas, Qureshi and Curtis, 1999; Millard et al., 1982; Clusin et al., 1982; Muller et al., 1988).

Given this, it is unsurprising that diltiazem and verapamil were unsuccessful in clinical trials. Verapamil failed to improve mortality over a 12 month period in one clinical trial (The Danish Study Group on Verapamil in Myocardial Infarction, 1984), and in a longer study of 18 months (The Danish Study Group on Verapamil in Myocardial Infarction, 1990). In both trials, SA or AV nodal arrhythmias were more common in those treated with verapamil and this led to treatment discontinuation (The Danish Study Group on Verapamil in Myocardial Infarction, 1984, 1990). Similarly, diltiazem did not improve mortality post-MI and doubled the risk of AV nodal block arrhythmias and hypotension (Moss et al., 1988). Therefore, treatment discontinuing ADRs were present even at subtherapeutic doses of verapamil and diltiazem, suggesting that drug doses could not be increased to achieve the desired therapeutic effect on mortality. Thus, available class 4 drugs are not appropriate for preventing ischaemia-induced VF due to their insufficient selectivity for the ischaemic myocardium, resulting in an unacceptably narrow TI.

1.3.2.5 Na^+ channel blockers

The Na^+ channel, $Na_v1.5$, which mediates I_{Na}, is a target for anti-VF drugs. As ischaemia progresses an increasing proportion of Na^+ channels are inactivated, leading to depression of I_{Na} and arrhythmogenic conduction slowing (Kléber et al., 1986; Buchanan, Saito and Gettes,

1985) prior to electrical quiescence (Hondeghem and Cotner, 1978). Potentiation of this I_{Na} inactivation by selective drug-induced Na^+ channel blockade in the IZ, or by targeting VF itself via rate-dependent block of Na^+ channels, may prevent VF by converting conduction slowing to conduction block, leading to hastened tissue inexcitability (Hondeghem, 1987; Hondeghem and Cotner, 1978). This antiarrhythmic mechanism is associated I_{Na} blocking drugs, referred to as class 1 drugs. In addition, given the increased magnitude of I_{NaL} during ischaemia (section 1.2.1.2) and the potential contribution of this to arrhythmogenesis (section 1.2.2.3), blockade of this current by I_{NaL} blockers may also serve to reduce the risk of ischaemia-induced arrhythmogenesis.

1.3.2.5.1 I_{NaL} blockers

When I_{Na} inactivates, either due to normal AP repolarisation or the sustained depolarisation characteristic of ischaemia, a small portion of the Na^+ current, I_{NaL}, persists throughout the AP plateau phase (Horváth et al., 2020). During ischaemia, the magnitude of I_{NaL} is increased and contributes to intracellular Na^+ accumulation (Saint, 2006). In response, intracellular Na^+ is extruded in exchange for Ca^{2+} by the Na^+/Ca^{2+} exchanger acting in reverse mode (Belardinelli, Shryock and Fraser, 2006). The resultant increase in intracellular Ca^{2+} may prolong APD, causing arrhythmias mediated by triggered activity (i.e. EADs or DADs) (Kistamás et al., 2021; Horváth et al., 2020; Antzelevitch et al., 2014). In addition, elevated cytosolic Ca^{2+} during ischaemia can impair ventricular contraction and relaxation (Belardinelli, Shryock and Fraser, 2006; Sossalla et al., 2011; Kistamás et al., 2021). Therefore, drug-induced I_{NaL} blockade may prevent the potentially deleterious effects of intracellular Na^+ and Ca^{2+} overload during ischaemia (Chaitman, 2006; Belardinelli, Shryock and Fraser, 2006). Ranolazine is a selective I_{NaL} blocking drug originally developed as an antianginal agent (Chaitman, 2006). In animal models of acute MI, ranolazine reduced the extent of ischaemic injury and improved LV contractile function (Gralinski et al., 1994) and, clinically, ranolazine has demonstrated beneficial effects in patients with CHD (Morrow et al., 2007). In the MERLIN-TIMI 36 trial, the incidence of recurrent ischaemia, worsening of angina and incidence of VT was significantly lower in those treated with ranolazine versus placebo (Morrow et al., 2007). However, in the same trial, the use of ranolazine had no effect on the rate of SCD or MI, and was associated

with higher rates of discontinuation of treatment due to ADRs (Morrow et al., 2007). Thus, I_{NaL} blockade represents a valid avenue for the treatment of angina and EADs/DADs in CHD but may not currently optimally target ischaemia-induced VF in order to effectively prevent SCD.

1.3.2.5.2 I_{Na} blockers: Class 1a vs 1b vs 1c

Na^+ channel blockers which block I_{Na} are divided into three subclasses, class 1a (e.g. quinidine), 1b (e.g. lidocaine and mexiletine) and 1c (e.g. flecainide), based upon their differing Na^+ channel association and dissociation kinetics, and the resulting electrophysiological consequences of these differences (Vaughan Williams, 1984, 1970; Lei et al., 2018). Class 1b drugs associate and dissociate quickly from the Na^+ channel, whereas class 1c drugs do so slowly, and class 1a drugs sit in between (Harrison, 1985). All class 1 agents bind preferentially to either the *A* or *I* state of the Na^+ channel and have low affinity for the *R* state (Hondeghem and Katzung, 1984). Class 1a drugs such as quinidine and class 1c drugs such as flecainide have relative selectivity for the Na^+ channel's *A* state (Kodama et al., 1987; Anno and Hondeghem, 1990), whereas class 1b drugs lidocaine and mexiletine have relative selectivity for the Na^+ channel's *I* state (Kodama et al., 1987; Matsubara, Clarkson and Hondeghem, 1987; Sunami et al., 1993). Membrane potential depolarisation causes the Na^+ channel to undergo conformational changes which facilitate the transition into its *A* and *I* states (section 1.3.1), and these structural changes reveal a high affinity drug binding site located in the Na^+ channel's pore (Pless et al., 2011). Site-directed mutagenesis has demonstrated that two residues present in the Na^+ channel's α subunit DIVS6 segment, phenylalanine (Phe1760) and tyrosine (Tyr1767), form an important part of the class 1 drug binding mechanism (Fozzard, Sheets and Hanck, 2011). However, due to differences in drug structure and pK_a, the class 1 drug subclasses may differ in the type and extent of interaction with this binding site (Nguyen et al., 2019; Liu, Atkins and Kass, 2003; Pless et al., 2011). For example, class 1b drug lidocaine depends primarily on a strong cation-pi interaction between its charged tertiary amine group and the aromatic group on the Phe1760 residue for drug binding which, alongside interaction with lidocaine's aromatic ring and the Tyr1767 residue, prevents ion conductance through the channel's pore (Pless et al., 2011). In contrast, class 1c

drug flecainide doesn't appear to rely on a cation-pi interaction with the Phe1760 residue (Pless et al., 2011) but, since the drug is large and branched in structure, flecainide forms more extensive molecular interactions within the channel pore (Nguyen et al., 2019), occupying a hydrophobic cavity between the DIIIS6 and DIVS6 segments wherein it makes contact with the Phe1760 residue (Salvage et al., 2018). Thus, all class 1 agents display some degree of use-dependent (Roden, Iansmith and Woosley, 1987; Bajaj et al., 1987; Kamiya et al., 1989; Matsubara, Clarkson and Hondeghem, 1987; Campbell and Vaughan Williams, 1983) and voltage-dependent Na^+ channel block (Hondeghem and Katzung, 1977, 1984), making them inherently tachycardia-selective and ischaemia-selective, but to varying extents, likely due to differences at the molecular level.

Due to their quick association and dissociation from the Na^+ channel, class 1b drugs do not affect myocardial conduction at sinus rate because there is a sufficiently long diastolic interval to allow drug-induced channel blockade acquired during systole to rapidly recover (with a time constant specific to each drug, influenced by membrane potential and pH) (Hondeghem, 1987). In contrast, since class 1c agents dissociate more slowly, there is a risk of residual channel blockade when the next AP arrives, even during sinus rhythm (Hondeghem, 1987). As a result, class 1b drugs display greater use-dependence of block than class 1c drugs, with class 1b effects revealed only at higher heart rates when the diastolic interval is short (Hondeghem, 1987). Together with their marked voltage-dependence (Hondeghem and Katzung, 1977), these properties afford class 1b drugs with superior ischaemia-selectivity and tachycardia-selectivity versus other class 1 drugs. The effect of class 1b antiarrhythmics is to decrease the time spent in the critical window of ischaemia-induced proarrhythmic slowing of conduction by hastened bidirectional conduction block in the ischaemic tissue (Hondeghem and Cotner, 1978; Hondeghem, 1987). It is also worth noting that lidocaine and mexiletine, whilst they preferentially block I_{Na}, also block I_{NaL} (Horváth et al., 2020; Antzelevitch et al., 2014) and therefore may also ameliorate the adverse effects of increased I_{NaL} during ischaemia (section 1.3.2.5.1).

1.3.2.5.3 Class 1a and 1c drug-induced proarrhythmia

Class 1a and 1c drugs have foundered in the treatment of ventricular arrhythmias. In the Cardiac Arrhythmia Suppression Trial (CAST), flecainide increased the risk of arrhythmogenic death by 3-fold, and more than doubled mortality, versus placebo in MI survivors (CAST Investigators, 1989). The underlying mechanism of proarrhythmia was suspected to result from a drug interaction with ischaemic tissue (Greenberg et al., 1995), exacerbating ischaemia-induced conduction slowing and differences in I_{Na} block between ischaemic and non-ischaemic tissue (Anderson et al., 1994), increasing the risk of re-entry. A meta-analysis evaluating the use of antiarrhythmic drugs after MI revealed that class 1a drugs also showed a trend towards excess mortality (Teo, Yusuf and Furberg, 1993), attributable to their APD prolonging effect (Imaizumi and Giles, 1987) which may provoke triggered activity mediated by EADs, resulting in TDP (section 1.2.2.3) (Levine, Morganroth and Kadish, 1989).

1.3.2.5.4 Class 1b drugs

Given the above, it is unsurprising that, of all class 1 drugs, only the class 1b drugs mexiletine and lidocaine are used in the treatment of ischaemia-induced ventricular arrhythmias, specifically in tandem with ICD implantation (Abboud and Ehrlich, 2016), for shock-refractory ventricular arrhythmias during SCA (Soar et al., 2019), and when an individual's risk of ventricular arrhythmias is very high (Dan et al., 2018; Joint Formulary Committee, 2019; Al-Khatib et al., 2018).

The use of mexiletine came after preclinical observations of its antiarrhythmic capability (Amerini et al., 1985; Igwemezie et al., 1992; Komori et al., 1994, 1995), and was substantiated by several small clinical trials which revealed its antiarrhythmic effect in patients with MI (Bell et al., 1982; Chamberlain et al., 1980; Campbell et al., 1975; Halinen et al., 1984) and benefit on mortality (Bell et al., 1982; Smyllie et al., 1984). However, the largest and longest trial investigating the use of mexiletine in patients post-MI, reported the drug to be ineffective at reducing mortality (IMPACT Research Group, 1984). Indeed, mexiletine's benefit on mortality is questionable (Chamberlain et al., 1980; Teo, Yusuf and Furberg, 1993), and, despite its class 1b properties, its tendency to induce ADRs mediated outside the IZ of the heart is high (Monk and Brogden, 1990), even at therapeutic doses (Rutledge, Harris and Amsterdam, 1985). The clinically effective plasma concentration range for mexiletine is 0.5-

2.0 μg/ml (i.e. 2.8-11.2 μM) (Campbell et al., 1978; Zipes and Troup, 1978). However, ADRs arise at plasma concentrations of approximately 0.88 μg/ml (i.e. 4.9 μM), and treatment withdrawal is common once the plasma concentration reaches 0.95 μg/ml (i.e. 5.3 μM) (Rutledge, Harris and Amsterdam, 1985), leaving its TI unacceptably narrow. Mexiletine's cardiovascular ADRs include negative inotropy (Honerjäger et al., 1986; Gottlieb and Weinberg, 1992; Rousson et al., 1986) and the capacity to adversely affect AV conduction (Satoh, Narimatsu and Taira, 1981; Lang, Just and Limbourg, 1975; Roos, Paalman and Dunning, 1976; Harper, Bertil Olsson and Varnauskas, 1977). Non-cardiac ADRs are also well documented (Monk and Brogden, 1990), the most common of which is gastrointestinal (GI) disturbances (Bell et al., 1982; Chamberlain et al., 1980; Smyllie et al., 1984; IMPACT Research Group, 1984), although effects on the central nervous system (CNS) have also been observed at clinically relevant doses (Igwemezie et al., 1992). Given these issues, mexiletine is not considered sufficiently safe for widespread use, particularly in larger lower SCD risk groups (Dan et al., 2018; Joint Formulary Committee, 2019). It is reserved only for those at the highest risk of VF and SCD, where other options for antiarrhythmic pharmacotherapy are unavailable (Dan et al., 2018; Joint Formulary Committee, 2019; Al-Khatib et al., 2018).

In patients experiencing shock-refractory ventricular arrhythmias during SCA (Soar et al., 2019), or at imminent risk of ischaemia-induced VF (i.e. during an AMI), lidocaine may be administered by trained staff (i.v. only) to provide immediate defibrillation and improve the chance of survival *en route* to hospitalisation (MacMahon et al., 1988; Herlitz et al., 1997), although this improved survival rate is not extended to hospital discharge (Herlitz et al., 1997; Kudenchuk et al., 2016). In addition, when lidocaine is administered in hospital shortly following AMI, there is no evidence that this improves short-term mortality (as assessed at 30 days) (Aj, Anand and Bangdiwala, 2015), and may even have an adverse effect (Sadowski et al., 1999). Indeed, it has been suggested that although lidocaine may reduce the risk of death from VF, it may simultaneously increase the risk of death from bradycardia or asystole, (MacMahon et al., 1988; Sadowski et al., 1999). In addition to bradycardia and other cardiovascular complications (i.e. hypotension and heart block), the toxicity of lidocaine, particularly in the CNS, is well recognised, with disturbances ranging from confusion, dizziness and drowsiness to seizures and respiratory depression (Pfeifer et al., 1976; Aj, Anand and

Bangdiwala, 2015). Therefore, the lack of mortality benefit afforded by i.v. lidocaine may be due in part to dose-limiting ADRs, rather than an inability to provide an antiarrhythmic effect. Complicating its use further, lidocaine is susceptible to 'first-pass' metabolism in the liver, and as a result has poor oral bioavailability (approximately 35%) and therefore must be administered i.v. in the hospital setting and is inappropriate for regular chronic use (Boyes et al., 1971). In this regard mexiletine is superior to lidocaine, with bioavailability after oral administration reaching approximately 90%, permitting its use orally outside the hospital setting (Monk and Brogden, 1990).

Studies to clarify lidocaine's action in anaesthetised rats, dogs and pigs with regional ischaemia found that i.v. lidocaine reduced the incidence of VF, but was bradycardic (Canyon and Dobson, 2004; Bergey, Nocella and McCallum, 1982). In rat isolated hearts with regional ischaemia, at a concentration representative of clinical peak unbound plasma levels, lidocaine was ineffective at reducing VF, although a higher concentration indicative of the human total blood concentration was antifibrillatory (Farkas and Curtis, 2002). In the same hearts, lidocaine intermittently lowered heart rate and prolonged the PR interval (the latter indicative of AV conduction delay) (Farkas and Curtis, 2002), demonstrating its inadequate ischaemia-selectivity.

In summary, lidocaine and mexiletine are antiarrhythmic and possess a degree of tachycardia-selectivity and ischaemia-selectivity as a result of their inherent class 1b properties, making them superior for use against ischaemia-induced VF in comparison to other class 1 antiarrhythmics, and the exemplar drugs in classes 2, 3 and 4, resulting in their continued use in high SCD risk individuals today. However, their ischaemia-selectivity is clearly insufficient to avoid ADRs mediated outside the IZ of the heart at therapeutic doses, limiting their widespread use. A novel antiarrhythmic drug with hopes of use in diverse SCD risk populations would require superior ischaemia-selectivity to that of existing class 1b drugs, whilst retaining their beneficial tachycardia-selectivity and antiarrhythmic effect.

1.4 The prodrug concept

Prodrugs are inactive drug molecules that are designed to be modified or metabolised in the body, either enzymatically or by another chemical reaction, resulting in the release of an active drug *in vivo* (Huttunen, Raunio and Rautio, 2011). Between the years of 2010-2015, approximately 13% of small molecular weight drugs approved by the FDA were prodrugs, and of all currently globally marketed drugs, prodrugs are estimated to account for 10% (Rautio, Kärkkäinen and Sloan, 2017). An active drug molecule with the correct structural properties to achieve the desired therapeutic effect at its target tissue site may not possess the required properties to allow the drug to either be effectively administered, to access the target tissue site once *in vivo*, or to have its therapeutic effect without the risk of systemic ADRs. These shortcomings can prevent widespread use of the affected drug. To tackle this, the 'prodrug concept' has been heavily utilised in drug development since the 1950s to overcome multiple use-limiting properties of active drugs in current use or under investigation. This specifically involves modifying a drug to an inactive version, which is converted to the active drug counterpart *in vivo*, to influence and improve drug properties such as formulation and administration, absorption, distribution, metabolism and excretion, and toxicity (Huttunen, Raunio and Rautio, 2011).

1.4.1 Hypoxia-activated prodrugs

Targeted drug delivery has been extensively reviewed over many decades in the development of anti-cancer drugs, with the aim of both improving the limited accessibility of drugs to the tumour tissue and reducing their systemic toxicity and intolerability (Poste and Kirsh, 1983; Freeman and Mayhew, 1986; Chari, 1998; Vasir and Labhasetwar, 2005). One method utilised in this area is the development of hypoxia-activated prodrugs (HAPs), also known as bioreductive prodrugs, of active cytotoxic agents that are inactive *in vivo* until reduced to the active drug moiety selectively under hypoxic conditions by endogenously expressed oxidoreductases (Mistry et al., 2017; Phillips, 2016). Most HAPs are the polar product of a two-electron N-oxidation of an active tertiary amine counterpart (Bickel, 1971).

Hypoxia is characteristic of many solid tumours and is considered a negative prognostic and predictive factor, contributing to the progression of aggressive and treatment-resistant tumours (Weinmann, Belka and Plasswilm, 2004). Indeed, many HAPs proposed as treatments in numerous cancers have entered clinical testing, with varying results (Mistry et al., 2017; Phillips, 2016). The general mechanism of HAPs is as follows. In the absence of oxygen HAPs undergo a two-electron reduction catalysed by either 1-electron or 2-electron reductase enzymes present in the target tissue, with multiple variations of this scheme having been developed (Mistry et al., 2017; Phillips, 2016). Oxygen-sensitivity of the prodrug reduction is conferred by, in the case of the 1-electron reductase mechanisms, the scavenging, by oxygen, of the received electron on the once-reduced prodrug, resulting in redox cycling of the prodrug. In the case of 2-electron reductase mechanisms, oxygen-sensitivity of the reduction process is the result the requirement of NADPH-dependent N-oxide reductase enzymes and haemoproteins in the reduction process, in which oxygen competes with the prodrug for the active site on the reduced haem group (Mistry et al., 2017; Wilson and Hay, 2011).

1.4.1.1 AQ4N

Analkylaminoanthraquinone (AQ4) is the active metabolite of its di N-oxide, AQ4N. AQ4N is inactive in the body, with poor affinity for DNA and poor inhibitory action against topoisomerase II, whereas AQ4 is a prominent and highly cytotoxic topoisomerase II inhibitor (Smith et al., 1997). Topoisomerase II inhibition is also the cytotoxic mechanism exploited by the successful anticancer drugs etoposide and doxorubicin (Nitiss, 2009). AQ4N is reduced by an oxygen-sensitive 2-electron reductase mechanism and is the most clinically advanced aliphatic N-oxide HAP (Mistry et al., 2017; Patterson and McKeown, 2000). The 2-electron reduction of AQ4N is catalysed by haemoproteins, both nitric oxide synthase and NADPH-dependent CYP450 (CYP3A, 1A and 2B subfamilies) isozymes, to produce the topoisomerase II inhibitor AQ4 (Patterson and McKeown, 2000; Patterson, 2002). AQ4N showed positive results in two phase 1 clinical trials. In patients with oesophageal carcinoma, no dose limiting toxic ADRs, and no maximum tolerated dose, could be found (Steward et al., 2007). Additionally, hypoxia-selective accumulation of therapeutically relevant concentrations of AQ4 was demonstrated in the tumours of cancer patients administered AQ4N (Albertella et

al., 2008). In line with this, AQ4N entered a phase 2 clinical trial in late 2006, although thus far no results have been published (clinicaltrials.gov/ct2/show/NCT00394628).

1.4.1.2 Lidocaine N-oxide

Clearly there have been extensive efforts to develop antiarrhythmic drugs against ischaemia-induced VF, particularly through the targeting of ion channels (section 1.3.2). However, the use of even the most suitable candidates (e.g. class 1b drugs mexiletine and lidocaine) is limited by systemic ADRs resulting in an unacceptably narrow TI (section 1.3.2). In some cases, the approaches utilised in antiarrhythmic drug development have involved maximising the voltage-dependence and use-dependence of the pharmacophore to optimise the drug's ischaemia-selectivity and tachycardia-selectivity. An alternative approach not yet explored is the development of antiarrhythmic prodrugs, whose pharmacological actions would be ischaemia-activated. The hypoxia inherent to acute myocardial ischaemia provides a justification for exploring the development of an antiarrhythmic HAP in analogy with AQ4N in the cancer therapeutics realm. Lidocaine N-oxide (LNO), a HAP of lidocaine, was developed in the expectation that such a compound would be pharmacologically inactive, and stable, *in vivo* prior to its hypoxia-activated conversion to lidocaine during myocardial ischaemia, thereby it would display antiarrhythmic effectiveness in the absence of ADRs mediated in the heart and elsewhere in the body (Tien, 1999).

As with other N-oxide compounds, LNO is formed from lidocaine through N-oxidation (Patterson et al., 1986; Craig and Purushothaman, 1970) (Figure 1.5). In many aspects, LNO is like AQ4N. Both compounds are tertiary amine N-oxides and share similarities with respect to their mechanism of reduction (Tien, 1999). LNO is reduced to lidocaine in the heart by a two-electron reduction process (Figure 1.5). This begins with a one electron reduction of a haemoprotein, catalysed by an NADPH-dependent P450 reductase. LNO becomes incorporated with the Fe^{II} atom in the reduced haem forming a haem[Fe^{II}]-LNO complex. The N-O bond in the LNO structure is cleaved, triggering the release of an oxy-ferryl haemoprotein and the reduced tertiary amine (lidocaine). A further electron reduction of the oxy-ferryl

haemoprotein and the addition of $2H^+$ results in the formation of a haem[Fe^{III}] and one molecule of water (Tien, 1999).

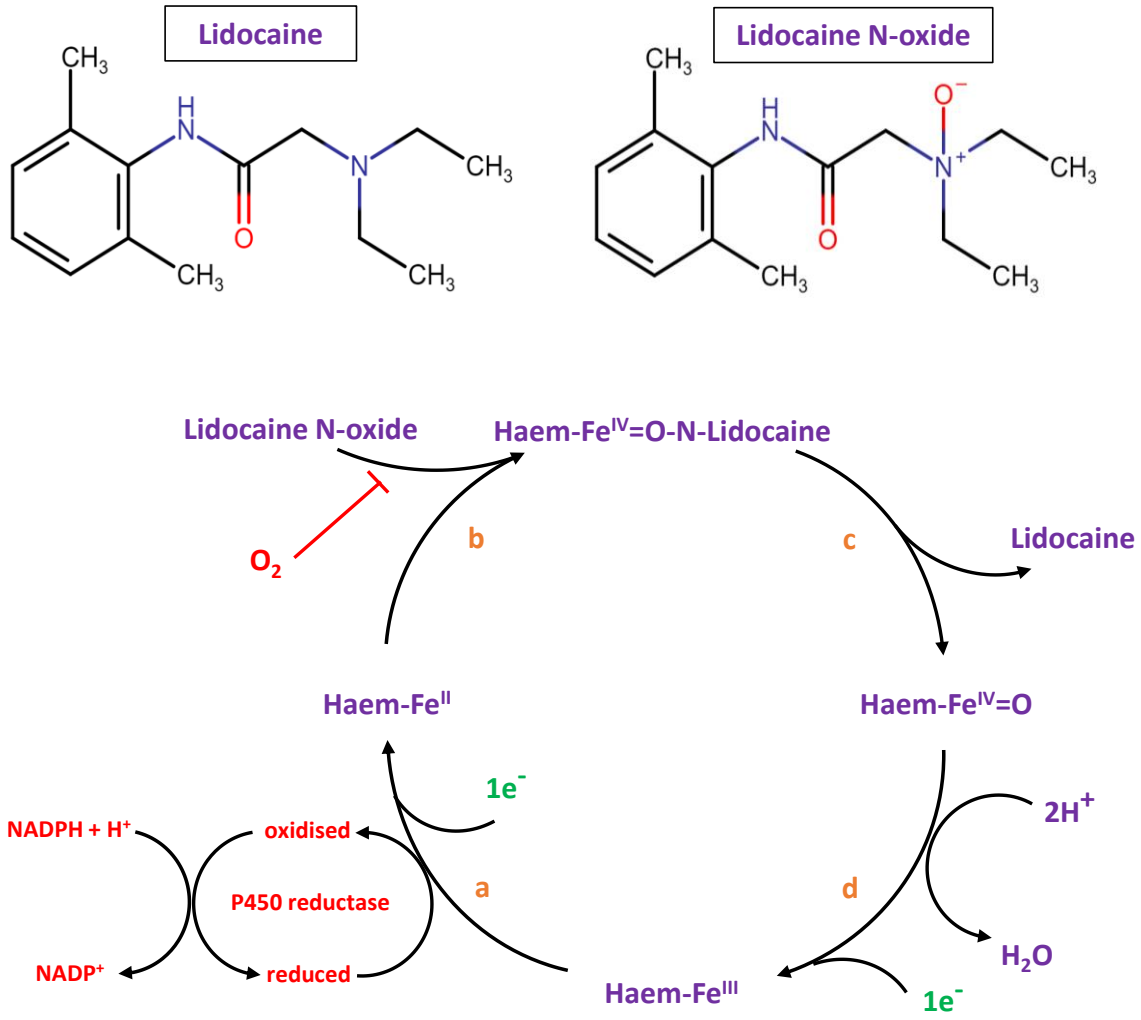


Figure 1.5 Chemical structures of lidocaine and lidocaine N-oxide and proposed mechanism of lidocaine N-oxide's reduction to lidocaine in the absence of oxygen. The mechanism begins with (a) a one electron reduction of a haemoprotein, catalysed by an NADPH-dependent N-oxide reductase, (b) with which lidocaine N-oxide becomes incorporated. After which, (c) the N-O bond is cleaved resulting in the release of an oxy-ferryl haemoprotein and lidocaine. Finally, (d) a further one electron reduction of the oxy-ferryl haemoprotein and the addition of $2H^+$ results in the formation of a haem[Fe^{III}] and water. This reduction is inhibited by the presence of oxygen. (O_2 = oxygen, $1e^-$ = 1 electron, H^+ = proton, H_2O = water). Figure adapted from Tien (1999) and Patterson (2002).

Preliminary *in vitro* experiments found that the reduction of LNO took place under anaerobic conditions in rat heart homogenate and S9 fraction, comprised of heart cytosol and SR,

wherein cytochrome P450 enzymes are plentiful, but not in heart sarcosomes (Tien, 1999). Thus, cytochrome P450 enzymes may be involved in LNO's reduction process. The precise isoform in the heart which may be involved was not identified (Tien, 1999). Additionally, other haemoproteins present in the myocardium were capable of catalysing LNO's reduction (Tien, 1999). Furthermore, the heat denaturation of rat heart S9 fractions diminished LNO's reduction by 40%, demonstrating the importance of enzymes/proteins in the reduction mechanism. However, the diminution and not abolition of LNO's reduction in this context suggested the existence of both an enzymatic and non-enzymatic mechanism available for the reduction of LNO (Tien, 1999). When investigating the oxygen-sensitivity of LNO's reduction in NADPH supplemented rat heart S9 fractions, the extent to which LNO levels depleted and lidocaine levels increased was regulated by the prevailing oxygen concentration (Tien, 1999). Indeed, as with other HAPs, the oxygen-sensitivity of the reduction of LNO was conferred by competition with oxygen for the active site in the reduced haemoprotein (Tien, 1999; Mistry et al., 2017; Wilson and Hay, 2011). Whilst LNO's ischaemia-activated reductive metabolism to lidocaine *in vitro* has been investigated, its antiarrhythmic effectiveness and mechanism of action has not been examined in *in vivo* and *ex vivo* models of acute myocardial ischaemia.

1.5 Hypothesis, aims and objectives

1.5.1 Hypothesis

LNO is expected to be pharmacologically inactive and stable in the normoxic myocardium but be reduced to lidocaine selectively in the ischaemic milieu. In line with this, LNO is anticipated to become commensurately pharmacologically active, with actions which mimic those of lidocaine and attributable to I_{Na} block, exclusively during myocardial ischaemia. This profile of drug action is proposed to afford LNO with the necessary ischaemia-selectivity to ensure the drug is devoid of ADRs and possesses a wide TI, offering a clear advantage compared with available antiarrhythmic drugs.

1.5.2 Objectives

The main objective was to examine whether LNO possesses ischaemia-activated antiarrhythmic activity against ischaemia-induced VF. This involved the characterisation of LNO's antiarrhythmic effectiveness in comparison to the occurrence of ADRs, the elucidation of LNO's antiarrhythmic mechanism of action focussing on actions attributable to I_{Na} block, and the demonstration of LNO's reduction to lidocaine in the ischaemic myocardium. Established models of ischaemia-induced VF were best utilised to achieve these goals.

Chapter 2: General experimental methods

A range of techniques were applied in different combinations throughout each part of the research strategy. Here the different techniques are described. The experimental protocols undertaken utilising these techniques, as well as additional rationale for protocol parameters, are detailed in the relevant subsequent chapters (3-5). All experiments were in compliance with the ARRIVE guidelines (Kilkenny et al., 2010) and the UK Home Office Guide on the Operation of the Animals (Scientific Procedures) Act 1986. All experiments were performed under an approved project license (PPL 70/7491) at King's College London, unless otherwise stated. Experiments were planned, undertaken and analysed in line with guidance from The Lambeth Conventions (Curtis et al., 2013) and The British Journal of Pharmacology's guidelines for experimental design and statistical analysis (Curtis et al., 2015).

2.1 Methods for rat Langendorff-perfused isolated hearts

The methods described here are well established, validated and have been extensively used (Wilder et al., 2016, 2018; Curtis, 1998; Andrag and Curtis, 2013; Clements-Jewery et al., 2006; Ridley, Yacoub and Curtis, 1992). Male Wistar rats (Envigo, UK) weighing between 290-500 g (specific weight ranges for each experiment are specified in the results chapters) were anaesthetised intraperitoneally (i.p.) with a lethal dose of sodium pentobarbitone (170 mg/kg), combined with 160 IU/kg heparin, an anticoagulant, to preclude the formation of blood clots in the coronary vasculature. A surgical level of anaesthesia was confirmed by the removal of the pedal and corneal reflexes, after which hearts was excised with the aortic arch still intact, causing death by exsanguination. Excised hearts were arrested by immediate submersion in ice-cold (4°C) Krebs buffer (NaCl 118.5 mM, $CaCl_2$ 1.4 mM, glucose 11.1 mM, $NaHCO_3$ 25.0 mM, $MgSO_4$ 1.2 mM, NaH_2PO_4 1.2 mM and KCl 3 mM). Langendorff-perfusion was initiated by cannulation via the ascending aorta, with Krebs perfusate delivered by a gravity-fed constant pressure system (approximately 80 mmHg). All perfusion solutions were made using water from a PURELAB Option Q or PURELAB Flex dispenser (ELGA LabWater, UK),

resistivity 18.2 MΩ, and were filtered (5 µm pore size), gassed with 95% O_2 and 5% CO_2 resulting in a pH of approximately 7.4, and subsequently warmed to 37°C prior to use. Krebs buffer was nominally normoxic (pO_2 = ~600 mmHg), allowing for normal heart function in the absence of haemoglobin (Yamada, Hearse and Curtis, 1990).

2.1.1 ECG placement and analysis

To assess cardiac rate and rhythm in the rat Langendorff-perfused isolated heart, an ECG trace recording was obtained by attaching two ECG leads to the perfusion cannula (earth and negative electrodes) and inserting a wire electrode into the heart 2 mm rostral to the apex on the LV surface. This was connected to a Powerlab system (Powerlab 4/35 and Animal Bio Amp, ADInstruments, UK; sampling rate 4 kHz) and connected to Labchart software (v.7, ADInstruments, UK). Changes in ECG morphology, indicative of changes in cardiac electrophysiology, were tracked through measurement of the PR interval (ms), QT interval at 90% repolarisation (QT_{90} interval) (ms) and heart rate (beats/min), extracted only when the heart was in sinus rhythm. If a heart was not in sinus rhythm at the exact time when measurements were due to be acquired, measurements were taken from the nearest preceding section of ECG displaying sinus rhythm, up to 1 min prior to the original timepoint.

2.1.2 Measurement of coronary perfusion

To measure coronary flow in the rat Langendorff-perfused isolated heart, coronary effluent was collected over timed intervals, at timepoints indicated by the relevant experimental protocol, and weighed (1 g = 1 ml). Values were denoted as ml/min/g to account for variations in heart weight.

2.1.3 Regional ischaemia via coronary artery ligation

To achieve a region of myocardial ischaemia, a needled silk suture (4-0, Ethicon) was sewn around the location of the left anterior descending (LAD) coronary artery, which originates adjacent to the left atrial appendage and runs down to the apex of the heart. The suture was threaded through a small piece of polyethene tubing and secured loosely in place with a pair

of curved Spencer Wells forceps. The suture was tightened, at a timepoint indicated by the relevant experimental protocol, by replacing the forceps tightly against the polyethene tubing, thereby preventing Krebs perfusion through the affected artery below the suture site. Regional myocardial ischaemia ensued, confirmed by observed changes to LV contractility and pallor. Reperfusion of the affected artery was achieved by subsequently loosening the suture.

2.1.3.1 Suture positioning

The size of the IZ formed by LAD coronary artery ligation in the rat Langendorff-perfused isolated heart is proportional to the resultant incidence of ischaemia-induced VF, in the absence of drug intervention (Ridley, Yacoub and Curtis, 1992; Wilder et al., 2016) (Figure 2.1 B). The placement of the coronary ligature along the length of the LAD coronary artery can be adjusted to produce varying IZ sizes when the suture is tightened. A ligation distal to the left atrial appendage, towards the apex, would produce an IZ size of approximately 20% of the total ventricular weight (TVW), whereas a ligation proximal to the left atrial appendage, approximately 1 mm below, would create an IZ of approximately 50% of the TVW (Ridley, Yacoub and Curtis, 1992; Andrag and Curtis, 2013; Wilder et al., 2016) (Figure 2.1A) ensuring approximately 15% or 75% of hearts in this experimental condition would develop VF, respectively (Ridley, Yacoub and Curtis, 1992; Wilder et al., 2016). For experiments undertaken in the completion of this thesis, either requiring a large scope for VF inhibition (i.e. investigating drug antiarrhythmic effectiveness) or a sufficiently large IZ surface area (i.e. investigating ventricular conduction), proximal LAD ligation was employed to ensure a large IZ was produced, and to maximise the likelihood of VF, in order to best investigate these variables.

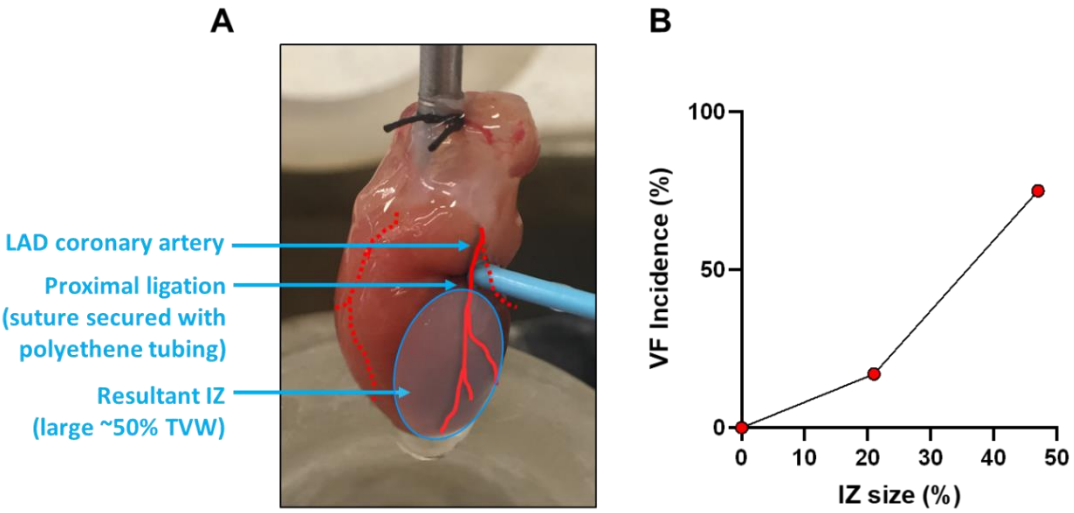


Figure 2.1 A) Labelled photograph of rat Langendorff-perfused isolated heart with a proximal ligation of the left anterior descending (LAD) coronary artery resulting in a large ischaemic zone (IZ) (~50% TVW). B) The incidence of ischaemia-induced ventricular fibrillation (VF) expected as a result of distal (~20% TVW) and proximal (~50% TVW) ligation of the LAD coronary artery. Figure adapted from Ridley et al. (1992).

2.1.3.2 Verification of IZ size

Upon cessation of experiments involving regional ischaemia, the size of the IZ was delineated by one of two methods, the blue dye method or the flow reduction method, to confirm parity of arrhythmogenic stimulus between experimental groups (Curtis and Hearse, 1989b). As per the former method, during reperfusion, with the ligature loosely in place, hearts were perfused with approximately 1 ml blue dye solution (1 mg/ml Patent Blue VF sodium salt, in 0.9% NaCl) before the ligature was re-tightened, trapping the dye in the IZ (Figure 2.2A). Perfusion with dye-free test solution was then resumed, allowing non-ischaemic tissue to clear of the blue dye, after which the IZ was visibly demarcated (Figure 2.2A). The IZ was then dissected from the non-IZ and both sections were weighed (Figure 2.2B). IZ size was then calculated as: IZ (%) = (IZ (g)/TVW (g))*100, wherein TVW (g) = IZ (g) + non-IZ (g) (Curtis and Hearse, 1989b). Alternatively, as per the latter method, following experiments from which myocardial tissue samples may be required for drug extraction (i.e. UHPLC-MS/MS analysis), contamination from the blue dye solution was prevented by quantifying IZ size by the percent reduction in coronary flow (ml/min/g) at +1 min after LAD ligation versus -1 min prior. This

method has been previously validated and reliably correlates to that of the blue dye method (Curtis and Hearse, 1989b).

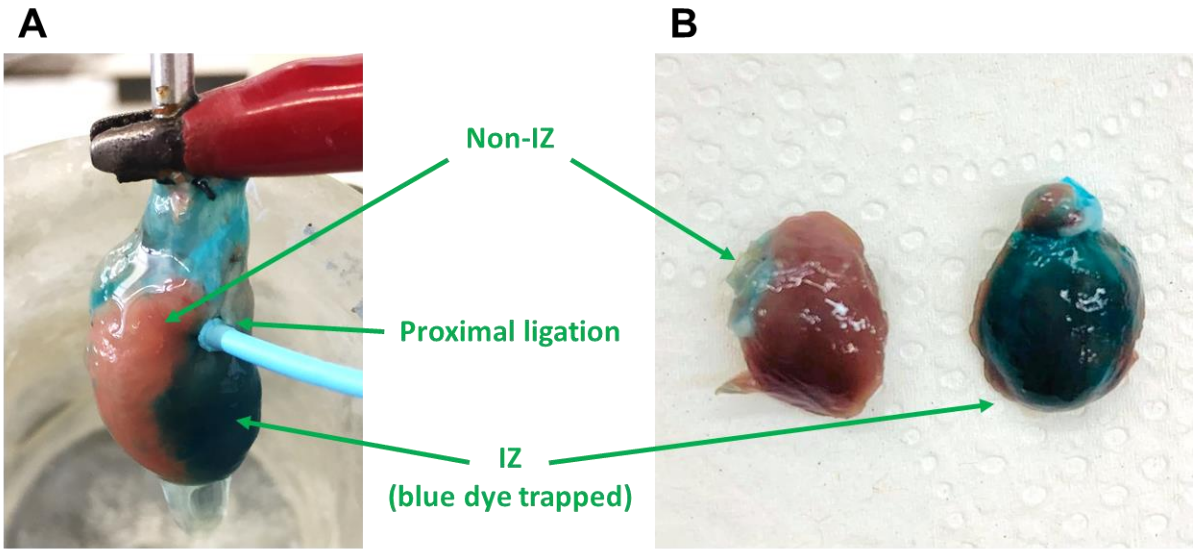


Figure 2.2 Labelled photographs of the ischaemic zone (IZ) and non-ischaemic zone (non-IZ) of A) a rat Langendorff-perfused isolated heart with proximal ligation of the left anterior descending (LAD) coronary artery and B) following IZ dissection.

2.1.3.3 Arrhythmia definitions and analysis

When arrhythmia incidence was investigated, the identification and analysis of arrhythmias was guided by the Lambeth Conventions (Curtis et al., 2013). Arrhythmias were classed as 'present' or 'absent' in sequential time periods throughout the relevant experimental protocol. The arrhythmogenic severity of the ischaemic period was also ranked by the assignment of an arrhythmia score (Table 2.1), permitting parametric statistical analysis, according to an established method (Wilder et al., 2016). In total, 5 pre-defined ventricular arrhythmias were investigated: ventricular premature beats (VPBs), bigeminy (BG), salvos (S), VT, non-sustained VF (NSVF) and sustained VF (SVF) (Figure 2.3).

Table 2.1 Arrhythmia scores

Score	*Arrhythmia*
0	VPB in 0 or 1 time period
1	VPB in >1 time period
2	BG or S
3	VT
4	NSVF (<120 s)
5	SVF (>120 s)

VPB = Ventricular premature beat, BG = Bigeminy, S = Salvos, VT = Ventricular tachycardia, NSVF = Non-sustained VF, SVF = Sustained VF

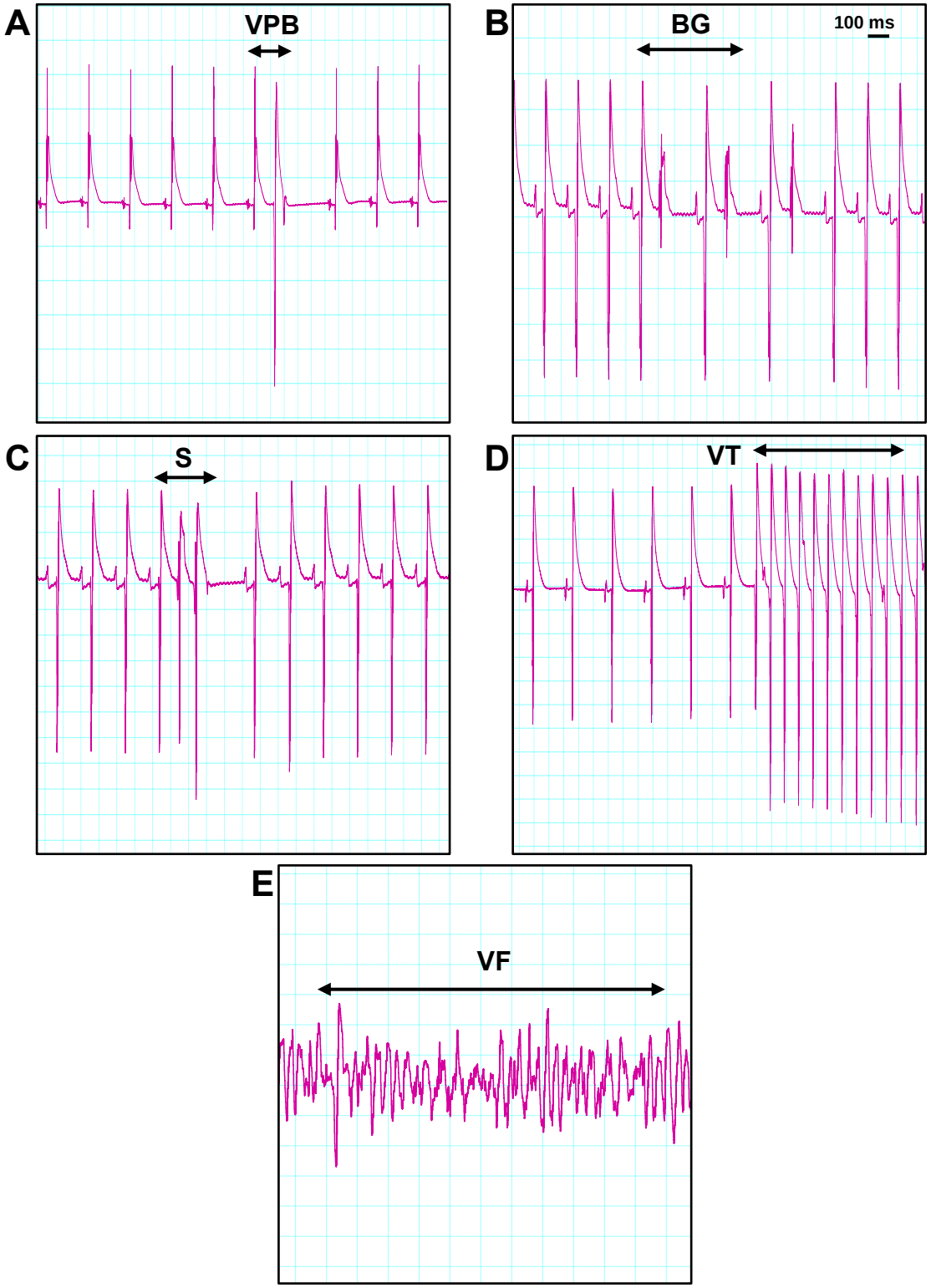


Figure 2.3 Examples of ventricular arrhythmias from regionally ischaemic rat Langendorff-perfused isolated hearts as defined by The Lambeth Conventions II (Curtis et al., 2013). A) ventricular premature beat (VPB), B) bigeminy (BG), C) salvos (S), D) ventricular tachycardia (VT) and E) ventricular fibrillation (VF). 1 box-width = 100 ms.

2.1.4 Global ischaemia via cessation of flow

In rat Langendorff-perfused isolated heart experiments that involved global ischaemia, this was achieved by clamping the Krebs inflow line, causing complete cessation of coronary flow. The heart was then immediately submerged in warmed Krebs (37°C) to maintain adequate tissue temperature and integrity for the duration of global ischaemia.

2.1.5 Assessment of contractile function via the intraventricular balloon (IVB)

When contractile function was assessed in rat Langendorff-perfused isolated hearts, a deflated intraventricular balloon (IVB), made from a compliant non-elastic material attached to a stiff plastic cannula, was positioned in the LV. This was achieved by incising the left atrium and gently inserting the IVB into the LV through the mitral valve until the tip of the IVB reached the apex of the heart (Wilder et al., 2016) (Figure 2.4A).

2.1.5.1 Pressure trace recording

The IVB and connective tubing was saline-filled and attached to a pressure transducer which was subsequently connected to Labchart software (v.7, ADInstruments, UK) and a Powerlab system (PowerLab 4/35 and Bridge Amp, ADInstruments, UK; sampling rate 4 kHz) to enable LV pressures to be recorded. The pressure transducer was calibrated daily using a sphygmomanometer prior to commencement of experimentation.

Before beginning the relevant experimental protocol, the 'zero' volume and 'working' volume of each heart were determined. The 'zero' volume was determined by minimally inflating the IVB with saline (~0.02 ml) until a pressure trace could just be detected. Using the 'zero' volume as a reference point, the IVB was then inflated with a large amount of saline (~0.12 ml for a 0.7-0.9 g rat heart) sufficient to produce a physiological level of LV stretch and generate a developed pressure of >100 mmHg, avoiding inducing a diastolic pressure >10 mmHg (to avoid excessive diastolic stretch) (Wilder et al., 2016). The volume required to achieve this was denoted as the 'working' volume and remained in the IVB for the duration

of the experimental protocol, unless a Starling curve (SC) was constructed (Wilder et al., 2016).

The systolic function and diastolic function of the hearts were assessed continually, at regular intervals indicated by the relevant experimental protocol, or by construction of a SC after an intervention. Measurements were only extracted when the heart was in sinus rhythm and at least 5 beats ahead from the cessation of any preceding arrhythmia. Diastolic pressure (mmHg) (i.e. the minimum value on the LV pressure trace) was used as a measure of diastolic function, and developed pressure (mmHg) (i.e. the difference between the minimum and maximum values on the LV pressure trace) was used as a measure of systolic function (Figure 2.4B). When indicated, a SC was constructed by deflating the IVB to its 'zero' volume before adding increments of 0.02 ml into the IVB up to a total of 0.14 ml, or until diastolic pressure was >20 mmHg, as per an established method (Wilder et al., 2016). Values for diastolic pressure and developed pressure were recorded at each 0.02 ml increment and plotted as pressure (mmHg) over IVB volume (ml). The Starling law describes the relationship between LV stretch (i.e. volume added to the IVB) and force of LV contraction (i.e. developed pressure generated) (Starling, 1918). A negative inotrope reduces the extent to which LV stretch generates increased contractile force, and thus reduces the SC slope, whereas a positive inotrope will achieve the opposite.

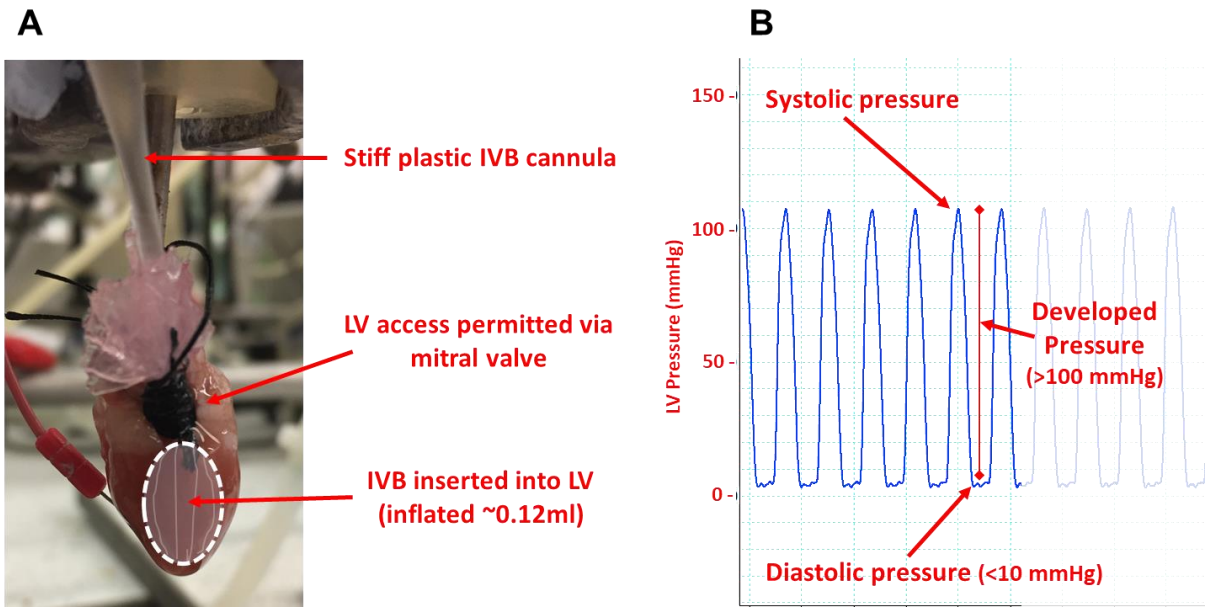


Figure 2.4 A) Labelled photograph of a rat Langendorff-perfused isolated heart with an intraventricular balloon (IVB) inserted into the left ventricle (LV) via the mitral valve, and a diagram of the placement of the IVB in the LV when inflated to 'working volume' with saline. B) The resultant baseline pressure trace labelled to indicate systolic (maximum) pressure (mmHg), diastolic (minimum) pressure (mmHg) and developed (maximum-minimum) pressure (mmHg).

2.1.6 Epicardial pacing via constant current stimulation

In the rat Langendorff-perfused isolated heart, when cardiac excitability and conduction experiments required the use of cardiac pacing, silver bipolar pacing wires were placed 2 mm rostral to the apex on the LV surface (Figure 2.5) The wires were attached to a DS3 Isolated Current Stimulator (DS3, Digitimer) and a PowerLab system (PowerLab 4/35 and Bridge Amp, ADInstruments, UK; sampling rate 4 kHz) connected to Labchart software (v.7, ADInstruments, UK). The current (mA) used to stimulate a heart during an epicardial pacing protocol differed based on the investigational endpoint (sections 2.1.6.1, 2.1.6.2 and 2.1.7), but in each case was determined based on the pacing threshold of the heart. The pacing threshold was the minimum current required to pace the heart at a frequency of 6.7 Hz (400 beats/min) and was determined 2 min prior to commencement of an epicardial pacing protocol by incrementally increasing the current supplied to the heart, from 0 mA until the heart begins to pace.

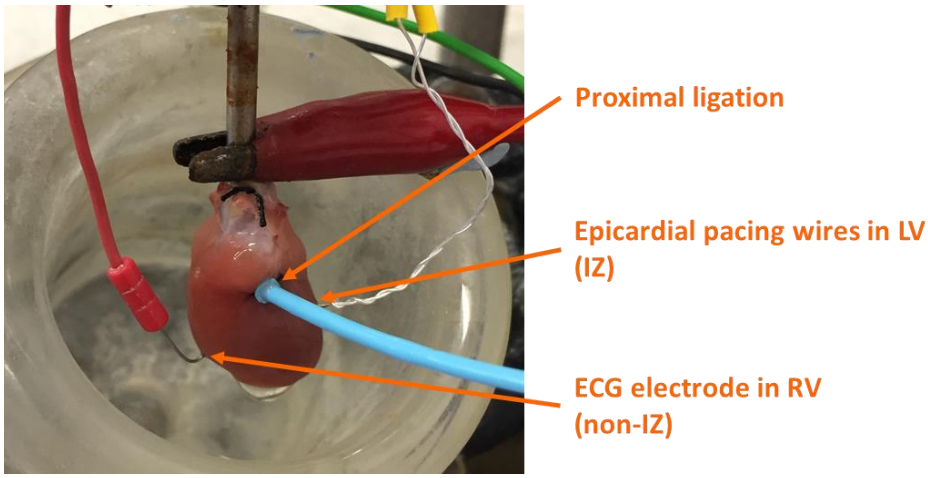


Figure 2.5 Labelled photograph of a rat Langendorff-perfused isolated heart with a proximal ligation of the left anterior descending (LAD) coronary artery, indicating the location of the bipolar epicardial pacing wires in the resultant ischaemic zone (IZ) of the left ventricle (LV) and an ECG electrode in the right ventricle (RV) (non-IZ).

2.1.6.1 Determination of effective refractory period (ERP) via epicardial pacing

When the effective refractory period (ERP) was assessed, rat Langendorff-perfused isolated hearts were set up with LAD coronary artery ligation (section 2.1.3), an ECG electrode (section 2.1.1) and epicardial pacing wires (Figure 2.5) in place. The ERP was determined by delivering a high constant current of 1.5 mA to hearts paced at 6.7 Hz (400 beats/min). This current was kept consistent across all hearts throughout the experimental protocol when the ERP was being investigated, irrespective of the diastolic pacing threshold. This was done for two reasons. Firstly, the ERP is current-dependent (i.e. a higher current stimulus strength will lead to the measurement of a shortened ERP). Secondly, accurately measuring the ERP during ischaemia is complicated since the diastolic pacing threshold constantly evolves, and the ERP is likely to change accordingly when using differing stimulus strengths (Opthof et al., 1993; Rozanski, Jalife and Moe, 1984). In addition, the ERP varies transmurally, particularly during ischaemia (Zhang et al., 2005), and therefore measurements were always taken from the epicardium. The choice of 1.5 mA was made based on a preliminary study in rat Langendorff-perfused isolated hearts perfused with Krebs or 15 µM lidocaine, in which the pacing

thresholds observed throughout normal perfusion and regional ischaemia ranged between 145 µA – 1.3 mA, with a mean ± SEM of 448 ± 46 µA (Figure 2.6). The mean pacing threshold was subsequently tripled to encapsulate all of the pacing thresholds recorded in the pilot study, and to therefore ensure the stimulus current used in the main study would be higher than the pacing threshold of all the hearts studied (i.e. all the hearts would pace).

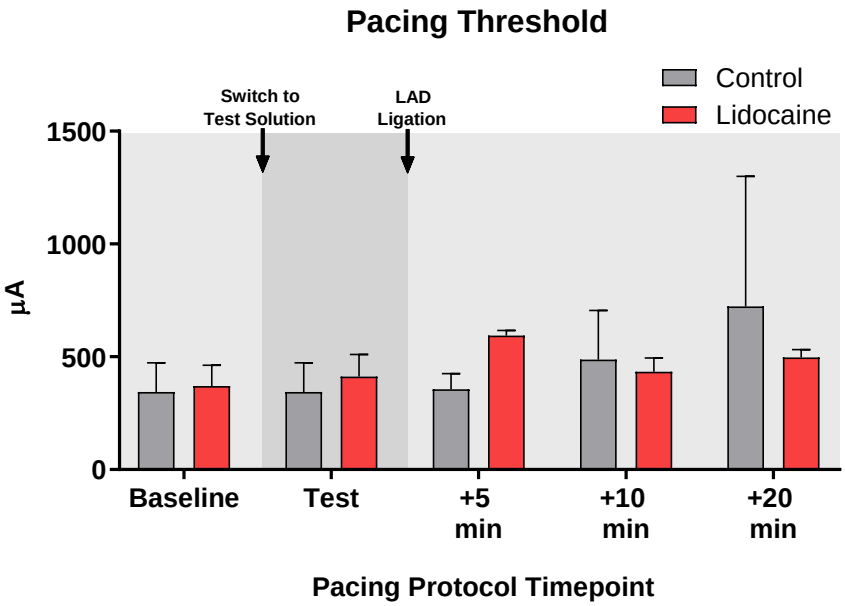


Figure 2.6 Pilot ERP study: Pacing threshold range. Determined in paced (6.7 Hz) isolated hearts perfused with Krebs or 15 µM Lidocaine during 20 min of regional ischaemia. n=3 per group; mean ± SEM.

The ERP was determined using a pre-programmed extra-stimulus (S1-S2) epicardial pacing protocol, consisting of a 40 beat S1 train (cycle length = 150 ms, 6.7 Hz) followed by a single S2, run continuously, with the S2 delivered at progressively shorter coupling intervals each run (10 ms step reductions from 110 ms) until loss of ventricular capture. At this point, S1 marked the beginning of phase 0 of the AP and S2 marked the longest interval from S1 that failed to result in subsequent depolarisation. This interval is the ERP (ms), and the above is an established method of its determination (Refsum and Landmark, 1976; Reiter, Synhorst and Mann, 1988; Barrabés et al., 2015).

2.1.6.2 Conduction time and conduction block via epicardial pacing

When conduction time and conduction block were examined, rat Langendorff-perfused isolated hearts were set up with LAD coronary artery ligation (section 2.1.3), epicardial pacing wires, and an ECG electrode (placed instead 2 mm rostral to the apex on the right ventricular surface) (section 2.1.1) in place (Figure 2.5). This arrangement ensured that the heart would be paced from in the IZ, and the ECG electrode was in non-ischaemic tissue, when regional ischaemia was induced. Once the pacing threshold for an individual heart was established, this value was doubled to determine the constant current used in that heart for the subsequent epicardial pacing protocols. In this, the constant currents used on each heart varied in line with the variation in each individual heart's pacing threshold. A pre-programmed epicardial pacing protocol was then run to investigate two separate endpoints, conduction time (ms) and conduction block. This pacing protocol consisted of 6 pacing rates (6.7, 8.3, 10, 11.7, 13.3 and 15 Hz), representative of increasing heart rates (400, 500, 600, 700, 800 and 900 beats/min), run for sequential 15 s time periods. The choice to use a range of pacing rates was made based on the knowledge that Na^+ channel blocking drugs have previously been shown to produce cumulative block during higher frequency rhythms (Matsubara, Clarkson and Hondeghem, 1987; Hondeghem and Katzung, 1984). The specific pacing rates used were chosen to represent a range encapsulating the normal sinus rate of a conscious rat (6.7 Hz, ~400 beats/min) (Schoemaker et al., 1990; Farmer and Levy, 1968), up to a pacing rate representative of rapid tachycardia or fibrillation (15 Hz, ~900 beats/min) (Ferdinandy, Das and Tosaki, 1993).

2.1.6.2.1 Conduction time analysis

Myocardial conduction time was defined as the time delay between the pacing stimulus and the subsequent paced ventricular beat (i.e. 'stimulus to QRS peak' interval) (Cascio et al., 1987). Due to the placement of the pacing wire, the pacing stimulus originated in the LV. Therefore, since the ECG electrode was placed in the right ventricle, the delay between the stimulus and the detection of a ventricular beat on the ECG was indicative of the time taken for the myocardium between the two points to propagate the electrical stimulus.

2.1.6.2.2 Conduction block analysis

Myocardial conduction block was defined as an instance in which the pacing stimulus, that originates from the pacing wires in the LV, was not followed by a resultant paced ventricular beat and is therefore blocked from propagating into an electrical signal conducted by the myocardium.

2.1.7 Measurement of conduction velocity through optical mapping

When voltage sensitive dye was used as an independent means of evaluating LV conduction velocity, rat Langendorff-perfused isolated hearts were imaged using an MVX10 microscope (Olympus) coupled to Evolve Delta 512 EMCDD cameras (Photometrics) and MetaMorph software. Data were sampled at 500 Hz with a spatial resolution of 390 μm^2 per pixel (64x64 pixels total). To allow for optimal positioning of rat Langendorff-perfused isolated hearts below the vertical camera, ensuring the LV was in the field of view, hearts were laid horizontally in a heated dish (~37°C). To achieve this, the descending aorta was cannulated *in situ* under pentobarbitone anaesthesia (170 mg/kg i.p.), and the heart was removed with the surrounding thoracic region of the spine, spinal cord and ribcage intact, as previously described (Winter et al., 2014) (Figure 2.7). Hearts were then Langendorff-perfused with Krebs buffer (constituents as described above in section 2.1) using a constant pressure pump system, and the subclavian and carotid vessels were clamped to prevent buffer leakage, maintaining a perfusion pressure of approximately 80 mmHg (Figure 2.7). The spinal column was then mechanically destroyed to preclude the influence of the sympathetic nervous system. Blebbistatin (15 µM), a mechanical uncoupling agent, was added to the Krebs perfusate reservoir and the voltage-sensitive dye, di-4-ANEPPs, was loaded into the heart via the perfusion line (40 µl, 1 mg/ml in DSMO) over several min. LAD coronary artery ligation was put in place (section 2.1.3), an ECG electrode was inserted into the right ventricular apex (section 2.1.1) and epicardial pacing wires were inserted as in section 2.1.6 (Figure 2.7). Hearts were paced at 6.7 Hz (400 beats/min) for the duration of the experimental protocol, at a current equivalent to double the pacing threshold of each heart. The LV was illuminated episcopically with an LED light source (excitation wavelength of 535 nm). Emitted light was

filtered through a 630 nm filter. Conduction velocity was calculated using the finite difference technique, as previously described (Cantwell et al., 2015).

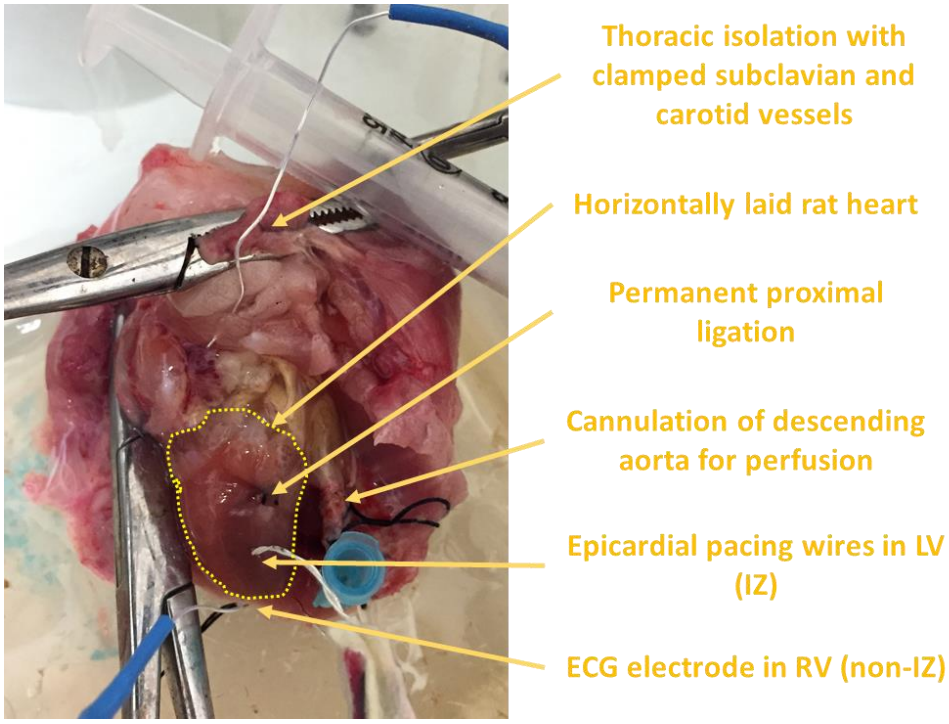


Figure 2.7 Labelled photograph of a rat Langendorff-perfused isolated heart (outlined in yellow dotted line) with cannulation of the descending aorta, laid horizontally across an isolation of the rat thoracic region with clamped subclavian and carotid vessels (to prevent Krebs leakage). Proximal permanent ligation of the left anterior descending (LAD) coronary artery is in place, with epicardial pacing wires inserted into the left ventricular (LV) ischaemic zone (IZ), and an ECG electrode inserted into the right ventricle (RV).

2.1.8 Langendorff exclusion criteria

Exclusion criteria for rat Langendorff-perfused isolated heart studies was implemented based upon previously published data in the model (Dhanjal et al., 2013; Clements-Jewery, Hearse and Curtis, 2002; Wilder et al., 2016). A heart was excluded if, during Krebs perfusion and prior to any intervention (i.e. before switch to test solution perfusate, induction of ischaemia or commencement of a pacing protocol), the coronary flow fell outside the range of <7 ml/min/g and >20 ml/min/g, or if the sinus heart rate was <200 beats/min. Hearts that were inappropriately arrhythmic prior to intervention were also excluded if this could not be

resolved. In experiments when regional ischaemia was induced (section 2.1.3), hearts were excluded if the IZ size, as measured by either the blue dye or flow reduction methods (section 2.1.3.2), was <35% or >60%, unless inclusion of the heart would not affect the outcome of the study (i.e. a heart with an IZ size of <35% with an occurrence of ischaemia-induced VF would not be excluded since the IZ size was clearly sufficient to still elicit VF) negating needless animal usage. Hearts that were excluded were subsequently replaced, whilst maintaining blinding, to ensure equal group sizes.

2.2 Methods for *in vivo* anaesthetised rats

Experiments using anaesthetised rats were undertaken at Imperial College London under the supervision of Dr. Markus Sikkel, who contributed significantly to the completion of these experiments. Due to the difference in institution, the experiments described in section 2.2 were performed under the project licence number PPL00/0776.

Male Sprague Dawley rats weighing 500-750 g (specific weight ranges for each experiment are specified in the results chapters), ex-breeders selected for their older age and larger size, were anaesthetised in an induction chamber using 5% isoflurane to allow for shaving of the chest, under arm areas, thighs and neck, and intubated via an endotracheal tube (blunted 14G i.v. cannula, Venflon). Intubated animals were attached to a Harvard Ventilator (Harvard Apparatus, Massachusetts, USA), and mechanically ventilated initially with 5% isoflurane at a rate of 90 breathes/min, with a tidal volume of 3 ml, before the isoflurane concentration was reduced to a maintenance level of approximately 1.5% for the remainder of the experiment. Isoflurane was the chosen anaesthetic since its use does not influence ischaemia-induced arrhythmia incidence or mortality (Jang, MacLeod and Walker, 1983). Rats were positioned and secured in the left lateral position on a warmed surface (to maintain body temperature at 37°C) and a 3-lead ECG was attached subcutaneously at the right thigh, right under arm and left lower abdomen. The ECG was connected to a PowerLab system (PowerLab 4/35 and Bridge Amp, ADInstruments, UK; sampling rate 4 kHz) using Labchart software (v.7, ADInstruments, UK). A 22G i.v. cannula (Venflon) was inserted into the right jugular vein for drug administration via a Standard Infusion Only Pump 11 Elite infusion pump (Harvard

Apparatus, Massachusetts, USA). Lidocaine and LNO solutions were prepared in Becton Dickinson 10 ml syringes, diluted to the desired concentration in medical grade saline (0.9% NaCl).

2.2.1 Induction of regional ischaemia via coronary artery ligation

When ischaemia-induced arrhythmias were examined *in vivo*, a thoracotomy at the 4th intercostal space was carried out, ensuring an optimal view of the left atrium, left atrial appendage and the heart's apex. Drug boluses were administered when required via right jugular vein cannulation. Boluses were 2 mg/kg LNO, 2 mg/kg lidocaine or the equivalent volume of saline, followed immediately by a continuous infusion of 0.5 mg/kg/min of the same drug which continued for the remainder of the protocol. This dosing regimen was selected based on an assessment of clinical data which states that a therapeutic blood concentration of lidocaine, in humans with a normal cardiac output, is achieved at these doses (Lie et al., 1974; Collinsworth, Kalman and Harrison, 1974). Ligation of the LAD coronary artery was performed using a 6-0 prolene suture (Ethicon) 1-2 mm below the corner of the left atrium to produce a large IZ, capable of inducing a high incidence of VF within 5 min of ischaemia onset (Curtis, 1998). Ischaemia was confirmed through observational changes in LV contractility and pallor. Animals alive at the end of the experimental protocol were culled by cervical dislocation followed by exsanguination.

2.2.1.1 Arrhythmia and ECG trace analysis

The occurrence of VF, in addition to overall survival and other arrhythmias (VPB, BG, S, VT), was monitored during 30 min of LAD coronary artery ligation. Arrhythmias were defined and used to assign each heart an arrhythmia score as in section 2.1.3.3. If VF occurred, up to 3 attempts at defibrillation were tried using a small custom-built rat defibrillator (Ordodi et al., 2006). If a rhythm incompatible with life, either sustained VF or asystole, was present 2 min after the 3rd defibrillation attempt then the animal was deemed to have not survived. Heart rate (beats/min) and PR and QT_{90} interval times (ms) were recorded as described in section 2.1.1 at the timepoints indicated by the relevant experimental protocol.

2.2.2 Pressure-volume (PV) loop catheterisation

When cumulative dose acute haemodynamic toxicity was examined *in vivo*, a left parasternal incision was made, and limited thoracotomy was performed in the 5th intercostal space. A 1.9F Scisense PV catheter was then inserted into the heart 2 mm rostral to the apex on the LV surface. Data were collected by an ADVantange acquisition system (Scisense Inc., Ontario, Canada) connected to Labchart software (v.7, ADInstruments, UK). Boluses of lidocaine or LNO were administered via right jugular vein cannulation in the amount of 1, 1, 2, 4, 8, 16 and 32 mg/kg at 2 min intervals, giving cumulative doses of 1, 2, 4, 8, 16, 32 and 64 mg/kg, respectively. These doses were chosen to identify LNO's dose threshold for adversity relative to that of lidocaine, for which supratherapeutic doses begin at approximately 4 mg/kg (Collinsworth, Kalman and Harrison, 1974). At the end of the experimental protocol, animals were culled by cervical dislocation and exsanguination.

2.2.2.1 PV loop and ECG trace analysis

PR and QT_{90} interval times (ms) and heart rate (beats/min) were recorded as described in section 2.1.1 at the timepoints indicated on the relevant experimental protocol. Cardiac output (ml/min) was calculated using the equation: Cardiac output = Heart rate x Stroke volume, in which Stroke Volume = End-systolic volume – End-diastolic volume. 10 PV loop cycles were averaged and analysed using the PV loop Analysis Module on Labchart (v.7, ADInstruments, UK).

2.3 Ultra-high performance liquid chromatography mass spectrometry (UHPLC-MS/MS)

2.3.1 Stock solution preparation

Stock solutions (1 mg/ml) of lidocaine, LNO and internal standard (IS) (lidocaine-d10 or 2-[bis(1,1,2,2,2-pentadeuterioethyl)amino]-N-(2,6-dimethylphenyl)acetamide)) used for construction of calibration curves were prepared in methanol and stored at -20°C. These stock

solutions were used to generate a 9 part calibration graph (0.1-250 ng/ml) or a 13 part calibration graph (0, 0.1-500 ng/ml) depending on the analysis protocol implemented (section 5.2).

2.3.2 Sample collection

When the presence of LNO and lidocaine in the ventricular myocardium was examined, samples of ventricular tissue were frozen after being collected from rat Langendorff-perfused isolated hearts, perfused with either 15 µM LNO or 15 µM lidocaine and subjected to a period of either regional ischaemia or global ischaemia (section 2.1). The perfusion protocol and methodological details varied based on the objective (Table 5.1). Corresponding samples of ventricular tissue were also collected (and frozen) from rat Langendorff-perfused isolated hearts subjected to a time-matched period of normoxic perfusion.

2.3.3 Sample preparation for UHPLC-MS/MS

Frozen tissue samples were cut to obtain portions of 20-30 mg in weight. Tissue samples were then transferred into individual round bottom Eppendorf tubes (2 ml) containing 990 µl of methanol with hydrogen peroxide (H_2O_2) (H_2O_2 concentration varied between protocols (Table 5.1)) and 10 µl IS (100 ng/ml in methanol), and were accurately weighed. These samples were moved into cold plates and homogenised using a TissueLyser II device for 30 s at 30 Hz before being centrifuged for 5 min at 14000 rpm at 4°C. 50 µl of each sample was then transferred into individual UHPLC-MS/MS injection vials containing 250 µl of 0.2% formic acid in water, subsequently vortexed and injected into the UHPLC-MS/MS system (20 µl injection volume).

2.3.4 Instrumentation and experimental conditions for UHPLC-MS/MS analysis

The UHPLC-MS/MS system consisted of a Waters Xevo TQS-micro triple quadrupole mass spectrometer with an electrospray ionisation source (operated in positive mode), coupled to an Acquity H Class LC system. Data was acquired using MassLynx V4.1 software. Separations

were conducted on a Waters ACQUITY UPLC™ BEH C_{18} column (2.1x50 mm, 1.7 µm), maintained at 40°C. Binary gradient profiles were developed using water (Optima™ LC-MS grade, Fisher) with 0.1% formic acid (LC-MS grade, Fisher) (solvent A) and methanol (Optima™ LC-MS grade, Fisher) with 0.1% formic acid (solvent B) at a flow rate of 200 µl/min. Separations were conducted under the following chromatographic: 100% solvent A for 0.5 min, decreased to 10% in 4.5 min and subsequently to 0% in 1 min, maintained for 1.9 min before being increased over 0.2 min to 100%. Column equilibration time was 3 min and the total run time was 10 min. The sample injection volume was 20 µl. Mass spectrometer parameters were as follows: capillary voltage 3.1 kV, desolvation temperature 600°C, cone gas flow 1 l/Hr, desolvation gas flow 1000 l/Hr and dwell time 46 ms per analyte. Analyte quantification was performed using the multiple reaction monitoring (MRM) method shown in Table 2.2.

Table 2.2 MRM parameters used in the quantification of lidocaine and LNO from myocardial tissue samples

Substance	*Retention time (min)*	*Precursor ion (m/z)*	*Product ion (m/z)*	*Collision energy (V)*	*Cone voltage (V)*
Lidocaine	2.9	235.2	86.0 qualifier	20	40
			58.0 qualifier	40	
Lidocaine N-oxide	3.2	251.1	86.0 qualifier	17	40
			120.1 qualifier	28	
IS	2.9	245.2	96.0 qualifier	20	40
			63.0 qualifier	40	

MEGX*	1.8	207.5	58.1 qualifier	15	25
			122.0 qualifier	20	

MEGX = Monoethylglycinexylidide
*MRM transition not included in all UHPLC-MS/MS analysis performed

2.4 Statistical analysis

Experimental design and analysis were performed in line with published guidelines (Curtis et al., 2013, 2015). Gaussian distributed variables were subjected to t-tests (two groups), 1 way ANOVA (three or more groups) or 2 way ANOVA (multiple variables) followed by either Dunnett's (comparison of groups to one control group), Tukey's (comparison of all groups to each other) or Sidak's (comparison of two groups with multiple points) post hoc tests (if F was significant). Binomially distributed variables (e.g. arrhythmia incidence) were compared using Fisher's exact test. Survival data were analysed using the log-rank (Mantel-Cox) test. Line gradients (e.g. SC analysis) were compared between groups using linear regression analysis. Statistical significance was set at $p<0.05$ for all analysis. All statistical analysis was performed on Graphpad Prism software (v.8).

2.5 Drugs and materials

All drugs and salts used for experiments in this thesis were of reagent grade (Table 2.3). Distilled water for solutions and buffers had a resistivity of 18.2 MΩ and was supplied by a PURELAB flex dispenser and PURELAB Option-Q (ELGA LabWater), except for use in UHPLC-MS/MS experiments in which Optima LC/MS Grade Water was used (Fisher Scientific, UK). LNO was synthesised from commercially available lidocaine according to an established method used to prepare tertiary amine N-oxides (Craig and Purushothaman, 1970), and shown was to be authentic and pure by nuclear magnetic resonance (NMR) and mass spectroscopy.

Table 2.3 Suppliers of drugs, salts and analytical grade solutions used

Supplier	*Drug/Salt/Solution*
Merck, UK	Lidocaine
	Patent Blue VF Sodium Salt
	Blebbistatin
	MEGX
	Lidocaine-(diethyl-d_{10})
Biotherics, UK	Lidocaine N-oxide
Fisher Scientific, UK	Optima LC/MS Grade Formic Acid
	Optima LC/MS Grade Water
	Optima LC/MS Grade Methanol
Invitrogen, UK	Di-4-ANEPPS
VWR International, UK	$CaCl_2 2H_2O$
	Glucose D (+)
	Methanol
	NaCl
	$NaHCO_3$
	$MgSO_4 7H_20$
	NaH_2PO_4
	KCl

Chapter 3: Characterisation of the effects of LNO on ischaemia-induced VF and ancillary variables

3.1 Introduction

Previous efforts to obtain a safe and effective drug to prevent ischaemia-induced VF have been unsuccessful (section 1.3.2), despite it being the leading cause of SCD in CHD (Kauppila et al., 2018). In clinical trials, some drugs (e.g. flecainide and D-sotalol) paradoxically increased mortality (CAST Investigators, 1989; Waldo et al., 1996), whilst others (e.g. verapamil and diltiazem) lacked effectiveness at doses which caused ADRs (The Danish Study Group on Verapamil in Myocardial Infarction, 1990; Moss et al., 1988). Indeed, ADRs at therapeutic doses are also a major concern with the few antiarrhythmics still used in this context, mexiletine and amiodarone (Goldschlager et al., 2007; Rutledge, Harris and Amsterdam, 1985), and thus these drugs may only be used in the patient population at highest risk of VF (Dan et al., 2018; Joint Formulary Committee, 2019; Al-Khatib et al., 2018).

In order to identify potentially useful novel antiarrhythmic drugs, it is therefore necessary to utilise animal models to estimate from the translational TI (TTI) (Muller and Milton, 2012) whether drugs may possess an adequately large TI in humans (Schneiderman et al., 1964). This can be attempted in the rat Langendorff-perfused isolated heart and *in vivo* in the anaesthetised rat (Curtis, 1998; Curtis, Macleod and Walker, 1987). Mexiletine was recently shown to possess no discernible TTI in the rat Langendorff-perfused isolated heart (Hesketh et al., 2020), providing a benchmark with which to compare the TTI of LNO, which is the focus of this chapter.

Several aspects of the rat heart make it suitable for this purpose. Larger animal species (e.g. rabbit, pig) may better resemble human cardiovascular physiology (Bers, 2002), however rats

are superior in terms of price and ease of use (Curtis, Macleod and Walker, 1987). Additionally, coronary collateral vessels are uniformly sparse in the rat heart, giving rise to an IZ of reproducible size when the LAD coronary artery is ligated (Maxwell, Hearse and Yellon, 1987), yielding a predictable time-course of VF (Curtis, 1998) and well-characterised dependence of VF on IZ size (Ridley, Yacoub and Curtis, 1992).

Additionally, given that AMI and SCD are often the first clinical manifestations of CHD (Kannel, Cupples and D'Agostino, 1987; Norris, 1998; Wong et al., 2019; John et al., 2012; Myerburg and Junttila, 2012), prophylactic antiarrhythmic drug therapy to prevent ischaemia-induced VF is often not possible, and first in human studies with a novel anti-VF drug are likely to involve administration after ischaemia onset (section 6.4.4). The ability of LNO to prevent VF after ischaemia onset can be examined utilising the rat Langendorff-perfused isolated heart, since it retains ≈6% of collateral flow after LAD ligation, insufficient to mitigate against severe ischaemia yet sufficient to permit post-ligation drug access (Maxwell, Hearse and Yellon, 1987; Curtis, MacLeod and Walker, 1984). Simultaneous monitoring of cardiac ADRs, additive to antiarrhythmic effectiveness in *ex vivo* and *in vivo* rat models, interrogates the breadth of LNO's TTI (Hesketh et al., 2020). Contractile function may be examined in the rat Langendorff-perfused isolated heart using an IVB (Henderson et al., 2013; Bell, Mocanu and Yellon, 2011), but in a separate study since the IVB is antiarrhythmic (Wilder et al., 2016). Obtaining preliminary *in vivo* cumulative dose toxicology data in the anaesthetised rat (without myocardial ischaemia) will refine the TTI of LNO by evaluating haemodynamic ADRs which can't be assessed during Langendorff perfusion (e.g. cardiac output and stroke volume) and ECG ADRs that cannot be assessed *in vivo* during myocardial ischaemia due to the high incidence of VF and early animal death.

3.2 Methods

The experiments in this chapter were completed using the general methods described in sections 2.1 and 2.2 and are summarised in Table 3.1. Specific details of the experimental protocols implemented in this chapter, and their rationale, are provided in this section. The

rats used in the rat Langendorff-perfused isolated heart pre-ligation administration study, post-ligation administration study and IVB study weighed 300-420 g, 290-390 g, 330-430 g, respectively. The rats used in the anaesthetised rat coronary artery ligation study and cumulative dose toxicity study weighed 530-730 g and 500-750 g, respectively.

Table 3.1 Summary of studies

Animal model	*Study*	*Details*	*Group sizes*	*Drug administration*
Rat Langendorff-perfused isolated heart	Pre-ligation administration study*	Vehicle control	n=12	Switch to test solution 10 min pre-ligation
		15 µM LNO		
		15 µM lidocaine		
	Post-ligation administration study	Vehicle control	n=20	Switch to test solution 10 min post-ligation
		15 µM LNO		
		15 µM lidocaine		
		60 µM LNO		
		60 µM lidocaine		
	IVB study	Vehicle control	n=12	Switch to test solution after first SC
		15 µM LNO		
		15 µM lidocaine		
Anaesthetised rat**	Coronary artery ligation study	Saline control	n=6	Bolus (i.v.) 5 min prior to ligation, followed by continuous infusion
		2 mg/kg LNO (+0.5 mg/kg/min)		
		2 mg/kg lidocaine (+0.5 mg/kg/min)		
	Cumulative dose toxicity study	1, 2, 4, 8, 16, 32 and 64 mg/kg LNO	n=5	Sequential escalating boluses (i.v.) administered at 2 min intervals

	1, 2, 4, 8, 16, 32 and 64 mg/kg lidocaine

*Experiment performed by Laura Mahoney-Sanchez during an undergraduate studentship
**Experiments in the anaesthetised rat were performed under the supervision of, and in collaboration with, Dr Markus Sikkel at Imperial College London

3.2.1 Studies in the rat Langendorff-perfused isolated heart

3.2.1.1 Pre-ligation administration study

Hearts were set up for Langendorff perfusion with a LAD coronary artery ligature and ECG electrode in place (section 2.1). Following a 5 min Krebs perfusion period, baseline measurements for heart rate, coronary flow and PR and QT_{90} intervals were taken (sections 2.1.1 and 2.1.2) at 5 min and 1 min before perfusate was switched to Krebs plus either a vehicle control, 15 µM LNO or 15 µM lidocaine (n=12/group). Measurements for heart rate, coronary flow and PR and QT_{90} intervals were then taken at 9 min, 5 min and 1 min prior to the ligature being tightened for a period of 30 min. A large IZ, approximately 50% of the TVW, was anticipated based on ligature position. Measurements of heart rate, coronary flow and PR and QT_{90} intervals were also taken at regular 5 min intervals throughout the 30 min duration of ischaemia. The occurrence of arrhythmias during ischaemia were recorded (section 2.1.3.3). At the end of the protocol, samples of LV tissue were taken and immediately frozen in liquid nitrogen in case further experimentation involving drug extraction was required. Therefore, IZ size was quantified using the flow reduction method (section 2.1.3.2). Hearts were then removed from the cannula and TVW (g) was measured (Figure 3.1).

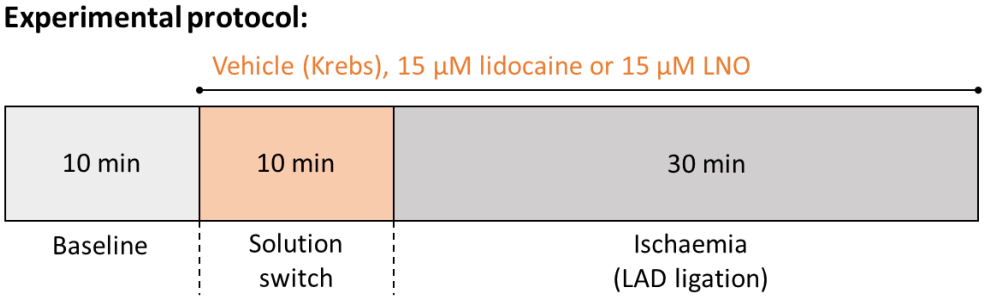


Figure 3.1 Diagram of the experimental protocol followed in the pre-ligation administration study.

The 30 min duration of regional ischaemia was chosen to examine the antiarrhythmic effects of each experimental group during peak susceptibility for phase 1 ischaemia-induced arrhythmogenesis, with the primary investigative variable being the occurrence of ischaemia-induced VF (Clements-Jewery, Hearse and Curtis, 2002; Curtis, 1998). The concentration of lidocaine and LNO was chosen based on a previous experiment in the rat Langendorff-perfused isolated heart model in which lidocaine was shown to prevent VF during regional ischaemia at a concentration of 12.93 µM (Farkas and Curtis, 2002), similar to the total blood concentration of lidocaine seen in humans at therapeutic dosage (Lie et al., 1974). The concentration of lidocaine was matched to that of LNO to allow direct comparison.

3.2.1.2 Post-ligation administration study

Hearts were set up for Langendorff perfusion with a LAD coronary artery ligature and ECG electrode in place (section 2.1). Following a 5 min Krebs perfusion period, baseline measurements for heart rate, coronary flow and PR and QT_{90} intervals were taken (sections 2.1.1 and 2.1.2) at 10 min, 5 min and 1 min prior to the ligature being tightened. A large IZ, approximately 50% of the TVW, was anticipated based on ligature position. Krebs perfusion continued until the 10^{th} min of ischaemia, upon which perfusate was switched to Krebs plus either a vehicle control, 15 µM LNO, 15 µM lidocaine, 60 µM LNO or 60µM lidocaine (n=20/group) for the remainder 20 min of ischaemia (30 min in total). Measurements of heart rate, coronary flow and PR and QT_{90} intervals were also taken at regular 5 min intervals throughout the 30 min duration of ischaemia, as well as 1 min prior to, and following, switch to test solution. Assessment of arrhythmias during ischaemia, measurement of IZ size and

collection of tissue samples were undertaken as before (section 3.2.1.1) and recorded (sections 2.1.3.2 and 2.1.3.3). Hearts were then removed from the cannula and TVW (g) was measured (Figure 3.2).

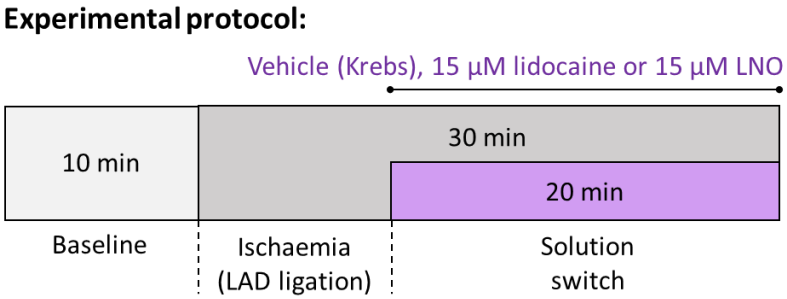


Figure 3.2 Diagram of the experimental protocol followed in the post-ligation administration study.

The duration of regional ischaemia and the 15 µM drug concentrations were chosen for the reasons described in section 3.2.1.1. Because IZ access and the effectiveness of LNO given post-ligation was unknown, a preliminary blinded and randomised pilot study was conducted using the above experimental protocol (Figure 3.2), with 15 µM, 30 µM and 60 µM LNO versus a vehicle control (n=3/group), which showed that 60 µM LNO abolished VF entirely (Figure 3.3). Thus, in addition to 15 µM lidocaine and 15 µM LNO groups, a 60 µM LNO group and a 60 µM lidocaine group were added to the full study, alongside a vehicle control. The choice to begin drug administration after the tightening of the ligature was made to test if LNO was effective given after ischaemia onset, since first in human studies are likely to involve drug administration after ischaemia onset (section 6.4.4). The choice to begin drug administration 10 min after ischaemia onset allowed for some scope for the drug to access the IZ before the risk of phase 1 VF peaks which, in a rat heart with a large IZ, is approximately 10-20 min after ischaemia onset (Curtis, 1998).

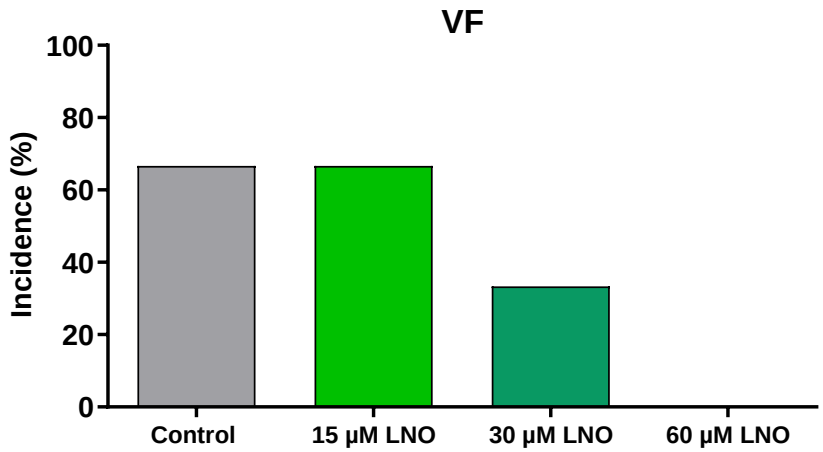


Figure 3.3 Pilot study to determine the concentrations to include in the post-ligation administration study. Concentration-dependence of LNO on VF incidence during 30 min of ischaemia in hearts perfused with Krebs, 15 µM LNO, 30 µM LNO or 60 µM LNO, with test solution perfusion commenced at 10 min after ligation. n=3 per group.

3.2.1.3 IVB study

Hearts were set up for Langendorff-perfusion with a LAD coronary artery ligature and an ECG electrode in place, and an IVB inserted into the LV (section 2.1). With the IVB inflated to its 'working' volume, hearts were perfused for 10 min with Krebs before a baseline SC was constructed (section 2.1.5.1). Values for developed and diastolic pressures were recorded at each 0.02 ml increment. After returning the IVB volume to its 'working' volume, perfusate was switched to one of three test solutions (vehicle control, 15 µM LNO and 15 µM lidocaine) for 5 min and then a second SC was constructed. An IZ of approximately 50% TVW was induced by tightening the ligature for a period of 30 min, and then the artery was reperfused for 60 min by loosening the ligature, after which a third SC was constructed. In the third instance, the IVB volume was increased to 0.14 ml without regard for any consequences of over-stretching the ventricle since this was the final part of the protocol and full inflation allowed full interrogation of ischaemia-induced diastolic dysfunction. Recordings of heart rate, coronary flow, PR and QT_{90} intervals (sections 2.1.1 and 2.1.2), developed pressure and diastolic pressure (section 2.1.5), were taken at regular 5 min intervals throughout the

protocol. At the end of each experiment, the IZ size was found by the blue dye method (section 2.1.3.2) (Figure 3.4).

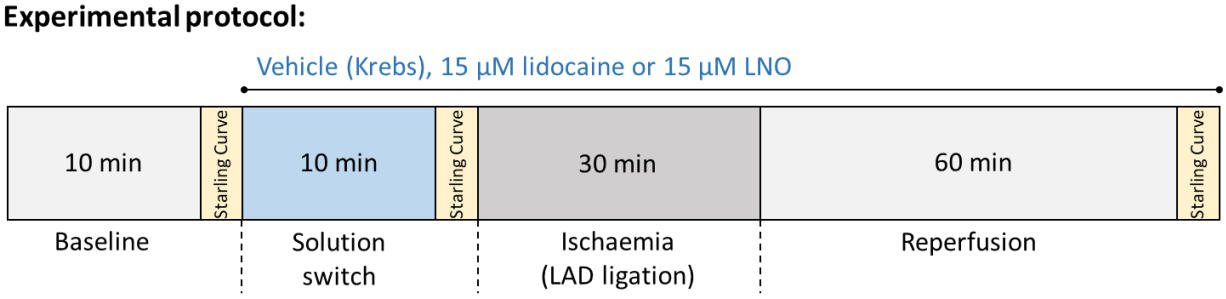


Figure 3.4 Diagram of the experimental protocol followed in the IVB study.

3.2.2 Studies in the anaesthetised rat

3.2.2.1 Coronary artery ligation study

Rats were anaesthetised, attached to an ECG monitoring system and the jugular vein was cannulated for drug administration as described in section 2.2. Following thoracotomy, baseline measurements of heart rate and PR and QT_{90} intervals were taken (section 2.1.1). Animals then received an i.v. bolus of either LNO, lidocaine or the equivalent volume of saline, followed immediately by a continuous infusion of the same drug which continued for the remainder of the protocol (section 2.2.1). Five min after administration of the drug bolus, measurements of heart rate and PR and QT_{90} intervals were again taken, after which hearts were made regionally ischaemic by a LAD coronary artery ligation (section 2.2.1). A large IZ, approximately 50% of the TVW, was anticipated based on ligature position. Ischaemia was induced for a total of 30 min as VF peaks approximately 5 min after ischaemia onset in this preparation and may persist for a further 20-25 min (Curtis, 1998), during which heart rate and PR and QT_{90} intervals were measured at 5 min intervals during sinus rhythm, and the occurrence of VF and death were recorded (section 2.2.1.1). Rats alive at the end of the protocol were culled by cervical dislocation and exsanguination (Figure 3.5).

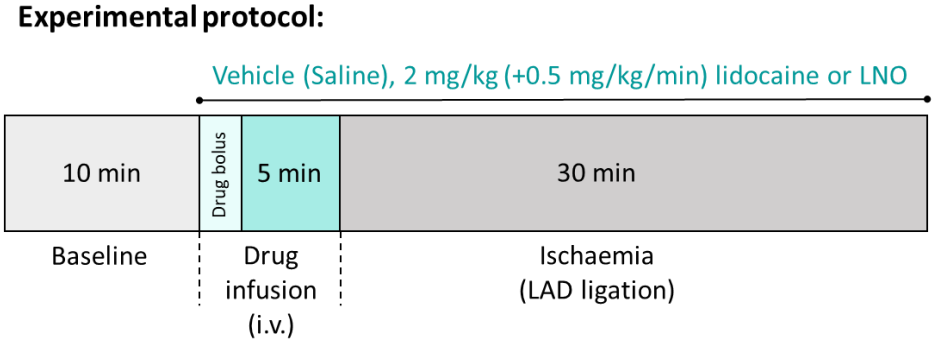


Figure 3.5 Diagram of the experimental protocol followed in the anaesthetised rat coronary artery ligation study.

3.2.2.2 Cumulative dose toxicity study

Rats were anaesthetised, attached to an ECG monitoring system and cannulated via the jugular vein for drug administration as described in section 2.2. Following thoracotomy, a PV catheter was inserted in the heart at the LV apex (section 2.2.2). Escalating boluses of lidocaine or LNO were administered as described in section 2.2.2. Recordings of heart rate, PR and QT_{90} intervals, as well as cardiac output and stroke volume (section 2.2.2.1), were taken 5 sec prior to administration of the next bolus. At the end of each protocol, animals were culled via cervical dislocation and exsanguination (Figure 3.6).

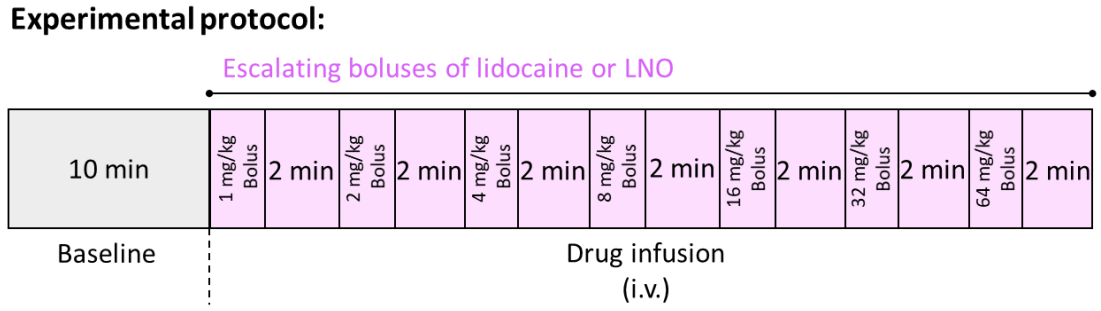


Figure 3.6 Diagram of the experimental protocol followed in the anaesthetised rat cumulative dose toxicity study.

3.3 Results

3.3.1 Studies in the rat Langendorff-perfused isolated heart

3.3.1.1 Pre-ligation administration study

3.3.1.1.1 Verification of experimental conditions

The mean IZ size did not differ between groups, as was expected, indicating randomisation methods were effective in establishing uniformity of arrhythmogenic stimulus (Figure 3.7).

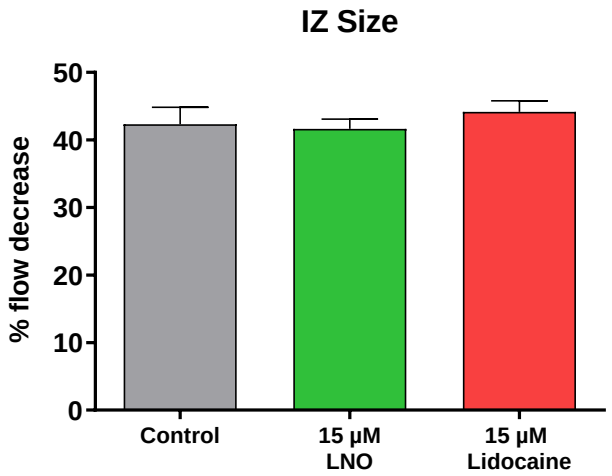


Figure 3.7 Langendorff-perfused isolated heart pre-ligation administration study: Verification of experimental conditions. IZ size in hearts perfused with Krebs, 15 µM LNO or 15 µM lidocaine. n=12 per group; mean ± SEM.

3.3.1.1.2 Arrhythmias

The incidence of VF in the control group was 60%, providing scope for inhibition of VF by LNO or lidocaine (Figure 3.8A). Lidocaine and LNO reduced VF incidence to the same extent (Figure 3.8A). The antiarrhythmic effect of LNO and lidocaine was also reflected by a reduction in the 30 min arrhythmia score (Figure 3.8B).

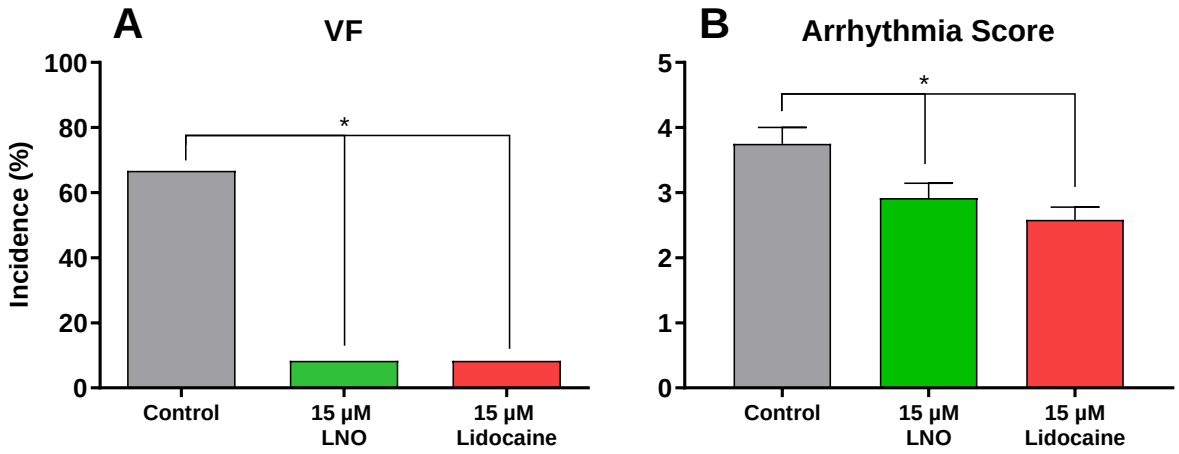


Figure 3.8 Langendorff-perfused isolated heart pre-ligation administration study: Arrhythmia incidence and score. A) Incidence of VF and B) arrhythmia score during 30 min of ischaemia in hearts perfused with Krebs, 15 µM LNO or 15 µM lidocaine. n=12 per group; incidence (A) or mean ± SEM (B). Binomially distributed variables (A) were compared using Fisher's exact test. Gaussian distributed variables (B) were subjected to 2 way ANOVA followed by Tukey's post hoc tests (following demonstration that F was significant and data Gaussian). *p<0.05 versus control.

3.3.1.1.3 Haemodynamic and ECG changes

There were no differences in heart rate, coronary flow or PR and QT_{90} interval between groups during baseline Krebs perfusion (Figure 3.9A-D). After switching to test solution perfusate, lidocaine caused bradycardia, whereas LNO did not (Figure 3.9A). The onset of ischaemia caused a small decrease in heart rate (Figure 3.9A), a small increase in QT_{90} interval (Figure 3.9D) and a large reduction in coronary flow (Figure 3.9B) in all groups, changes typical of those seen in previous publications utilising the preparation (Clements-Jewery et al., 2006; Wilder et al., 2016; Dhanjal et al., 2013; Farkas and Curtis, 2003; Hesketh et al., 2020). Throughout ischaemia, lidocaine continued to cause bradycardia (Figure 3.9A), as well as PR interval (Figure 3.9C) and QT_{90} interval (Figure 3.9D) prolongation. LNO had no effect on any variable at any timepoint (Figure 3.9A-D).

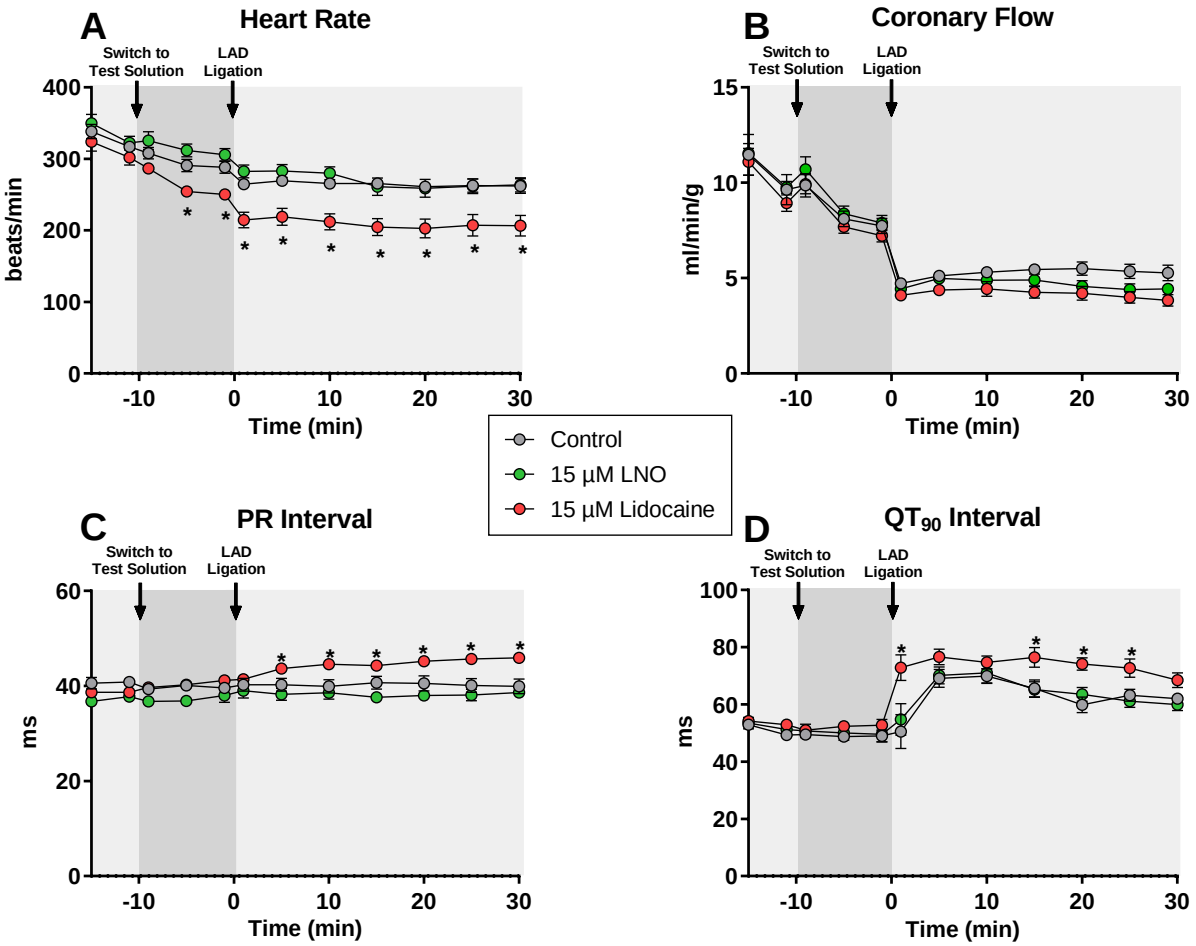


Figure 3.9 Langendorff-perfused isolated heart pre-ligation administration study: Haemodynamic and ECG changes. A) Heart rate, B) coronary flow, C) PR and D) QT_{90} intervals during 30 min of ischaemia in hearts perfused with Krebs, 15 µM LNO or 15 µM lidocaine. n=12 per group; mean ± SEM; variables were subjected to 2 way ANOVA followed by Tukey's post hoc tests (following demonstration that F was significant and data Gaussian). *p<0.05 versus control.

3.3.1.2 Post-ligation administration study

3.3.1.2.1 Verification of experimental conditions

The mean IZ size did not differ between groups, as was expected, indicating randomisation methods were effective in establishing uniformity of arrhythmogenic stimulus (Figure 3.10).

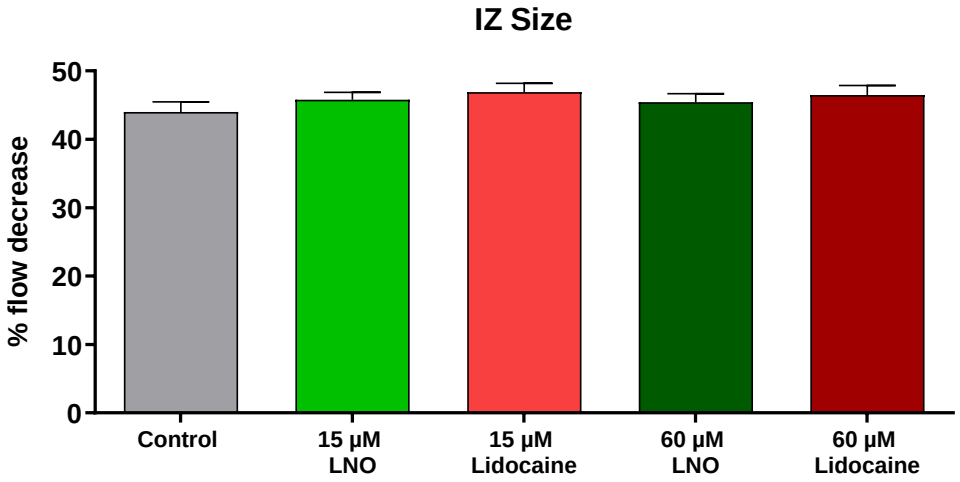


Figure 3.10 Langendorff-perfused isolated heart post-ligation administration study: Verification of experimental conditions. IZ size in hearts perfused with Krebs, 15 µM LNO, 15 µM lidocaine, 60 µM LNO or 60 µM lidocaine. n=20 per group; mean ± SEM.

3.3.1.2.2 Arrhythmias

The incidence of VF in the control group was 60%, providing scope for detection of inhibition of VF by LNO or lidocaine (Figure 3.11A). Lidocaine reduced VF incidence at 15 µM and 60 µM (Figure 3.11A). LNO was effective at 60 µM, but not 15 µM (Figure 3.11A). The arrhythmia score did not differ between groups during the first 10 min of ischaemia, indicating arrhythmia severity was similar across all groups before test solution perfusion began (Figure 3.11B), but was reduced by LNO and lidocaine during the full 30 min duration of ischaemia (Figure 3.11B).

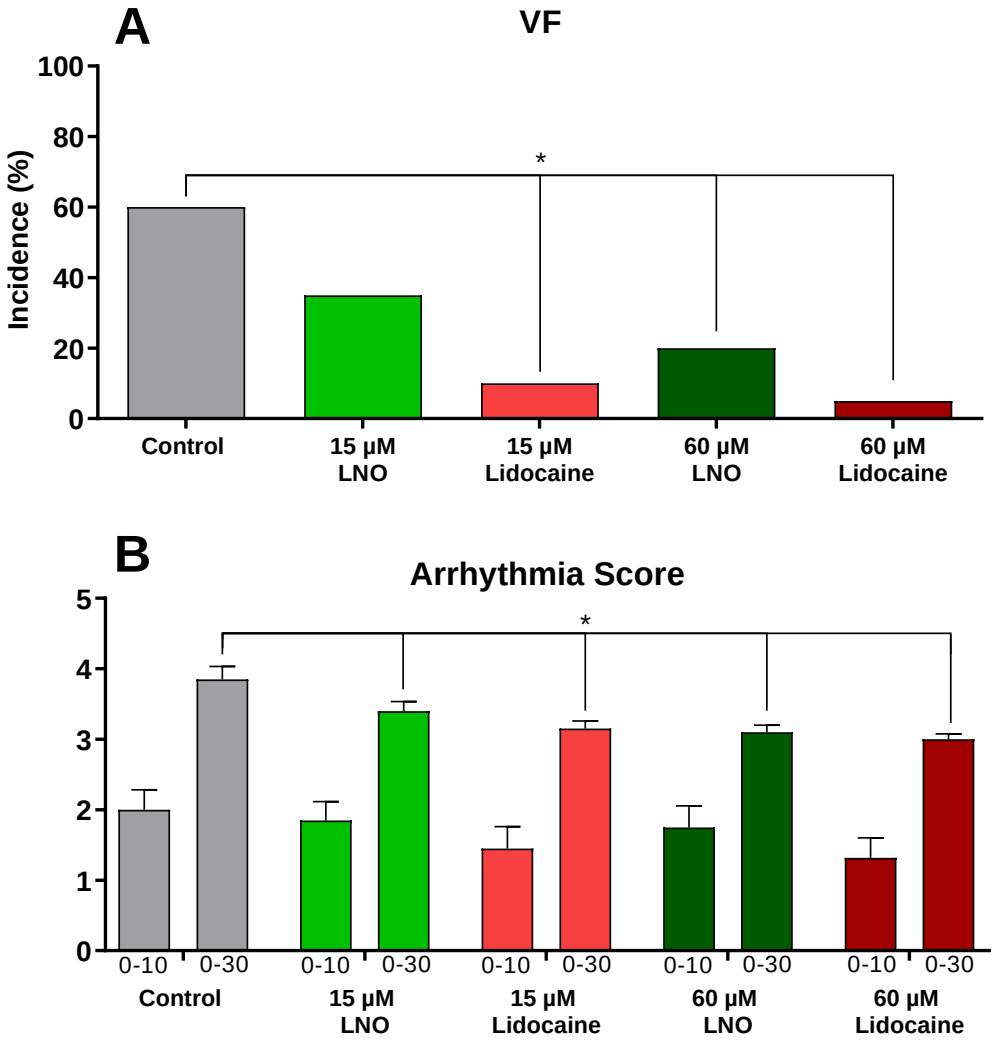


Figure 3.11 Langendorff-perfused isolated heart post-ligation administration study: Arrhythmia incidence and score. A) Incidence of VF and B) arrhythmia score during 30 min of ischaemia in hearts perfused with Krebs, 15 µM LNO, 15 µM lidocaine, 60 µM LNO or 60 µM lidocaine, with test solution perfusion commenced at 10 min after ligation. Arrhythmia Score is subdivided into 0-10 min and 0-30 min time bins. n=20 per group; incidence (A) or mean ± SEM (B). Binomially distributed variables (A) were compared using Fisher's exact test. Gaussian distributed variables (B) were subjected to 2 way ANOVA followed by Tukey's post hoc tests (following demonstration that F was significant and data Gaussian). *p<0.05 versus control.

3.3.1.2.3 Haemodynamic and ECG changes

There were no differences between the groups with respect to heart rate, coronary flow or PR and QT_{90} interval during baseline Krebs perfusion, or during the first 10 min of ischaemia (Figure 3.13A-D). Ischaemia onset was associated with a small decrease in heart rate (Figure 3.13A), a small increase in QT_{90} interval time (Figure 3.13D) and a large decrease in coronary flow (Figure 3.13B) in all groups, changes which are typical of the preparation (Clements-Jewery et al., 2006; Wilder et al., 2016; Dhanjal et al., 2013; Farkas and Curtis, 2003; Hesketh et al., 2020). Lidocaine caused bradycardia (Figure 3.12C, Figure 3.13A) and PR interval prolongation (Figure 3.12E, Figure 3.13C) at 15 µM and 60 µM, concentration-dependently. 60 µM lidocaine also decreased coronary flow (Figure 3.13B). LNO had no effect on any variable at either 15 µM or 60 µM (Figure 3.12B+D, Figure 3.13A-D).

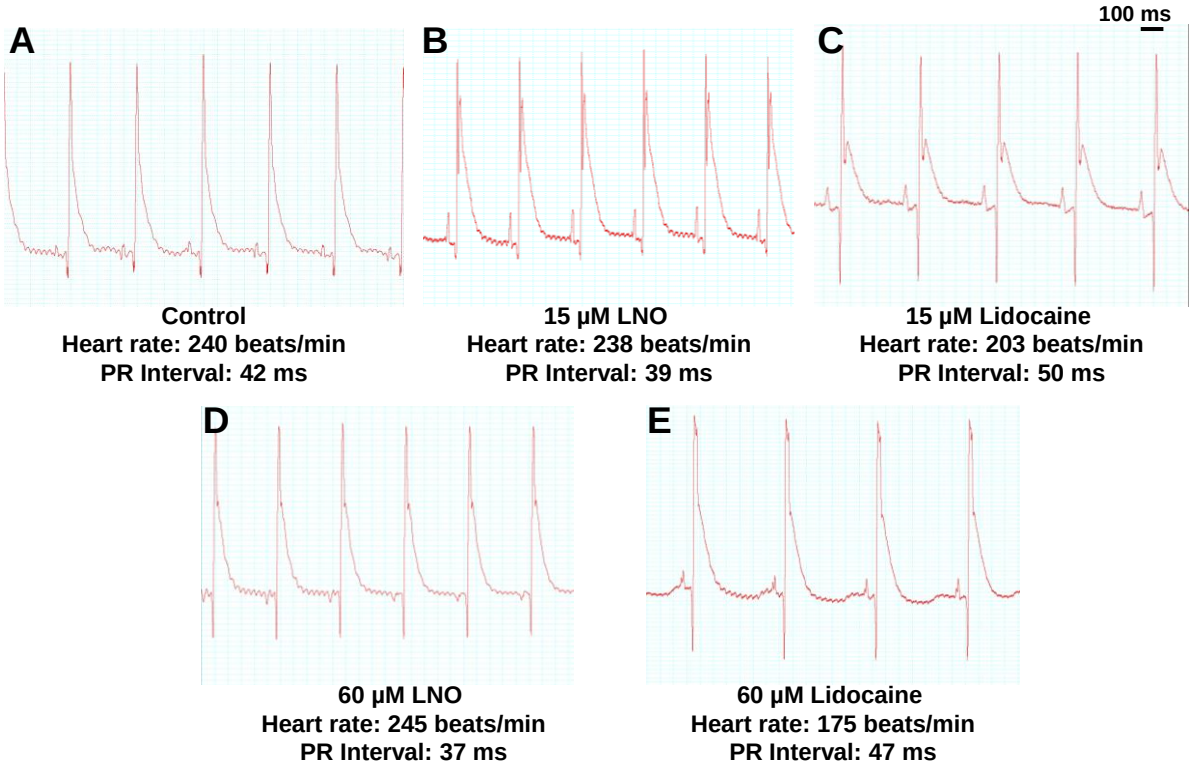


Figure 3.12 Langendorff-perfused isolated heart post-ligation administration study: Representative ECG traces. Representation of the differences between groups in heart rate and PR interval measurements taken 5 min after switch to A) Krebs control, B) 15 µM LNO, C) 15 µM lidocaine, D) 60 µM LNO or E) 60 µM lidocaine, with test solution perfusion commenced at 10 min after ligation. 1 box width = 50 ms.

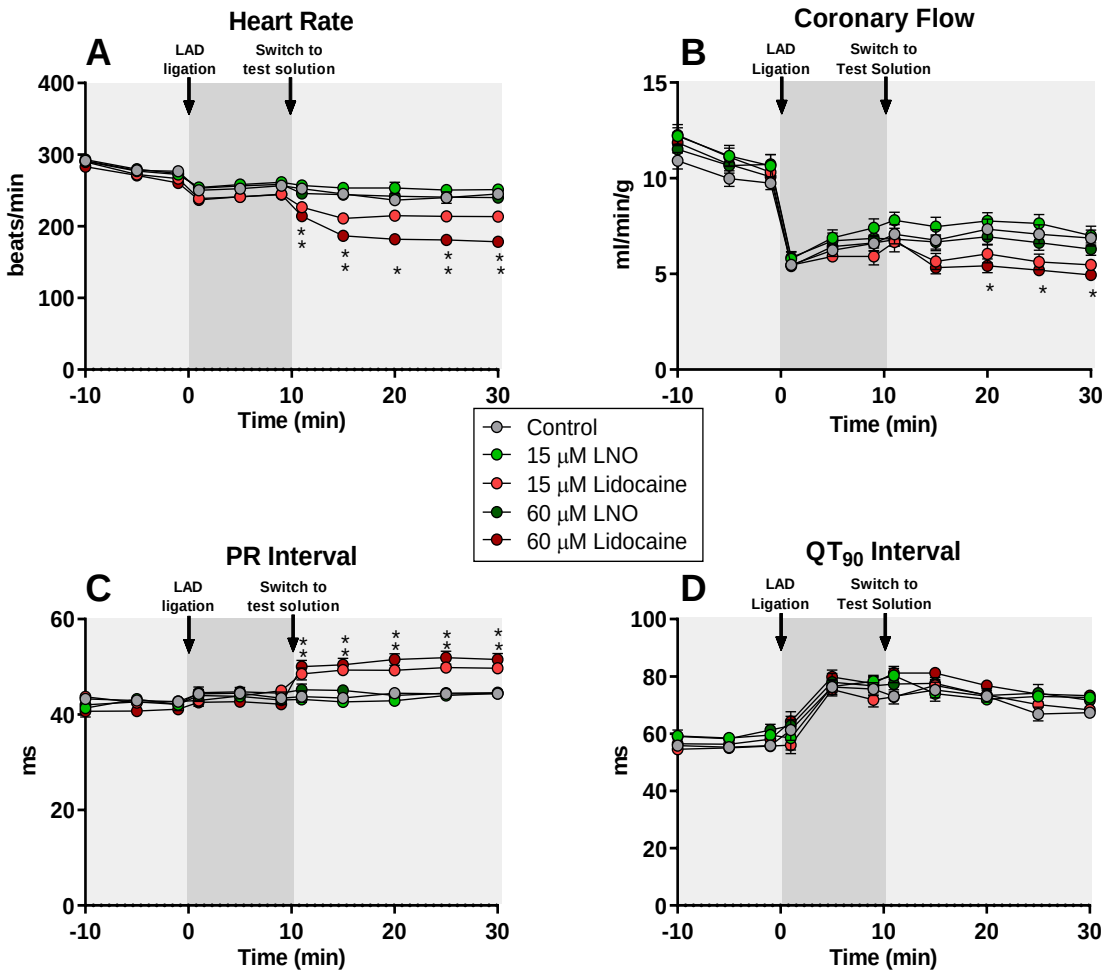


Figure 3.13 Langendorff-perfused isolated heart post-ligation administration study: Haemodynamic and ECG changes. A) Heart rate, B) coronary flow, C) PR and D) QT_{90} intervals during 30 min of ischaemia in hearts perfused with Krebs, 15 µM LNO, 15 µM lidocaine, 60 µM LNO or 60 µM lidocaine, with test solution perfusion commenced at 10 min after ligation. n=20 per group; mean ± SEM; variables were subjected to 2 way ANOVA followed by Tukey's post hoc tests (following demonstration that F was significant and data Gaussian). *p<0.05 versus control.

3.3.1.3 IVB study

3.3.1.3.1 Verification of experimental conditions

The mean IZ size was similar between groups (Figure 3.14).

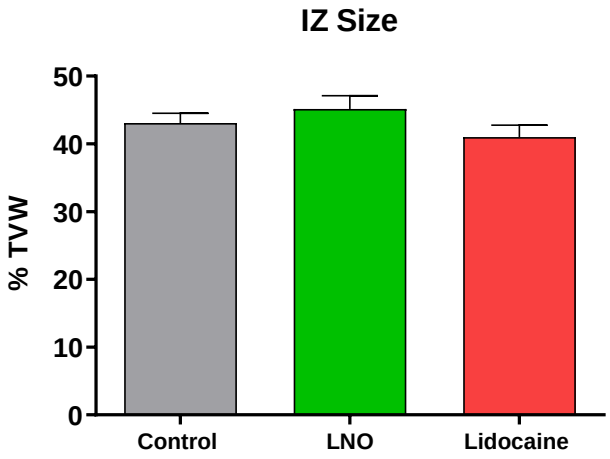


Figure 3.14 Langendorff-perfused isolated heart IVB study: Verification of experimental conditions. IZ size in hearts perfused with Krebs, 15 µM LNO or 15 µM lidocaine. n=12 per group; mean ± SEM.

3.3.1.3.2 Haemodynamic and ECG changes

There were no differences between groups in developed or diastolic pressures during baseline Krebs perfusion (Figure 3.15A+B). Developed pressure fell in response to regional ischaemia and failed to recover to 'pre-ischaemia' values during reperfusion, with the pattern similar in all groups (Figure 3.15A). Diastolic pressure increased slightly (by 2-5 mmHg) in response to ischaemia but increased approximately 3-fold (up to 30-40 mmHg) during reperfusion in all groups (Figure 3.15B). Recovery of diastolic function during reperfusion was poor, and appeared to reach a steady state by 60 min in all groups (Figure 3.15B). LNO appeared to increase diastolic pressure at 20 and 25 min into reperfusion, although this effect arose midway through reperfusion and was not maintained (Figure 3.15B). Therefore, neither lidocaine nor LNO had any consistent effect (beneficial or adverse) on systolic or diastolic function (Figure 3.15A+B).

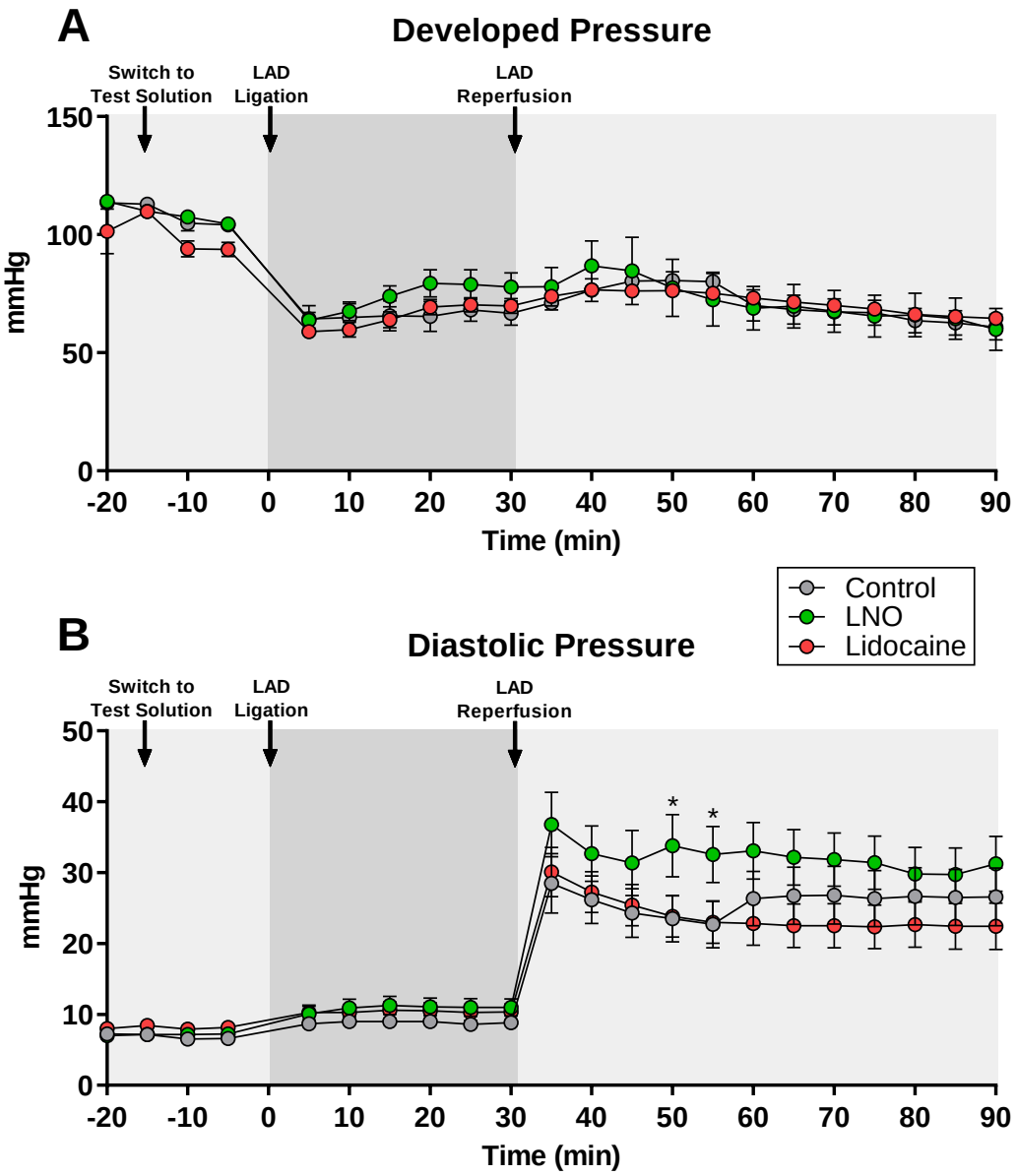


Figure 3.15 Langendorff-perfused isolated heart IVB study: Developed and diastolic pressure changes. A) Developed and B) diastolic pressures during 30 min of ischaemia followed by 60 min of reperfusion in hearts perfused with Krebs, 15 µM LNO or15 µM lidocaine. n=12 per group; mean ± SEM; variables were subjected to 2 way ANOVA followed by Tukey's post hoc tests (if F values were significant and data Gaussian). * $p < 0.05$ versus Krebs control.

Chapter 3: Characterisation of the effects of LNO on ischaemia-induced VF and ancillary variables

Heart rate, coronary flow, PR or QT_{90} intervals did not differ between groups during baseline Krebs perfusion (Figure 3.17A-D). Ischaemia evoked a decrease in heart rate (Figure 3.17A) and coronary flow (Figure 3.17D) and an increase in QT_{90} interval (Figure 3.17C) in all groups, as seen in the previous studies (section 3.2.1.1, 3.2.1.2) and in published work utilising this model (Clements-Jewery et al., 2006; Wilder et al., 2016; Dhanjal et al., 2013; Farkas and Curtis, 2003; Hesketh et al., 2020). Reperfusion was associated with recovery of coronary flow to 'pre-ischaemia' values in all groups (Figure 3.17D). No differences in QT_{90} interval were found between groups (Figure 3.17C). Lidocaine had no effect on coronary flow (Figure 3.17D) and although LNO appeared to increase coronary flow within the first 20 min of reperfusion, this effect was not maintained (Figure 3.17D). Lidocaine caused bradycardia and PR interval prolongation before, during and after ischaemia, whereas LNO had no effect on any ECG variable at any time during the protocol (Figure 3.16B+C, Figure 3.17A+B).

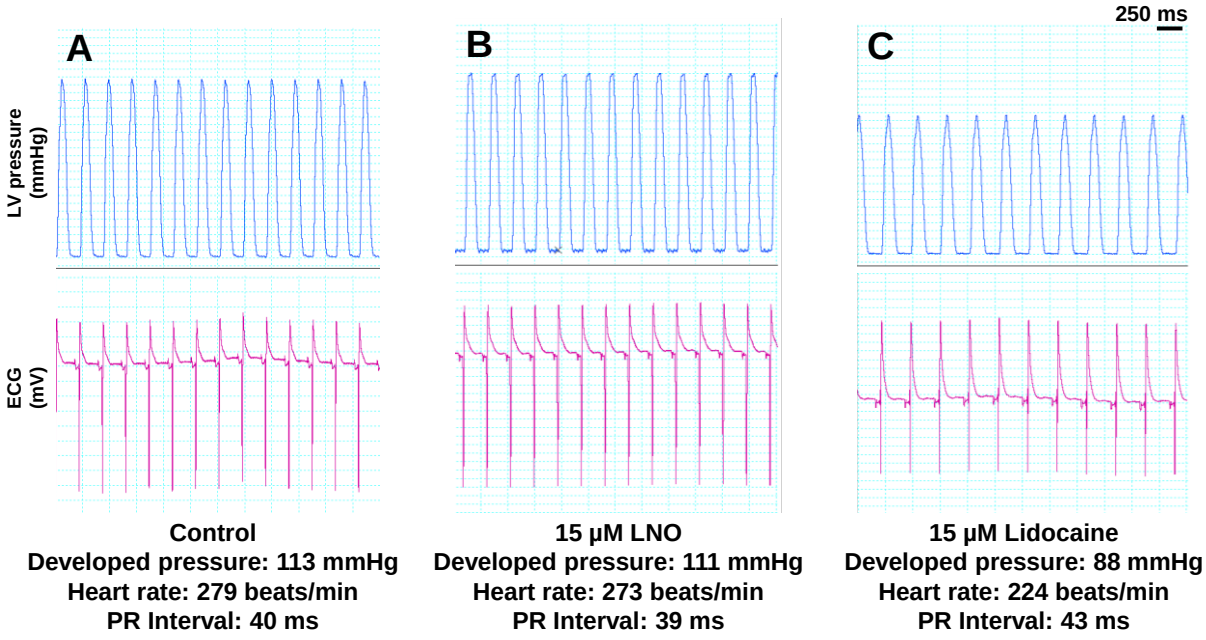
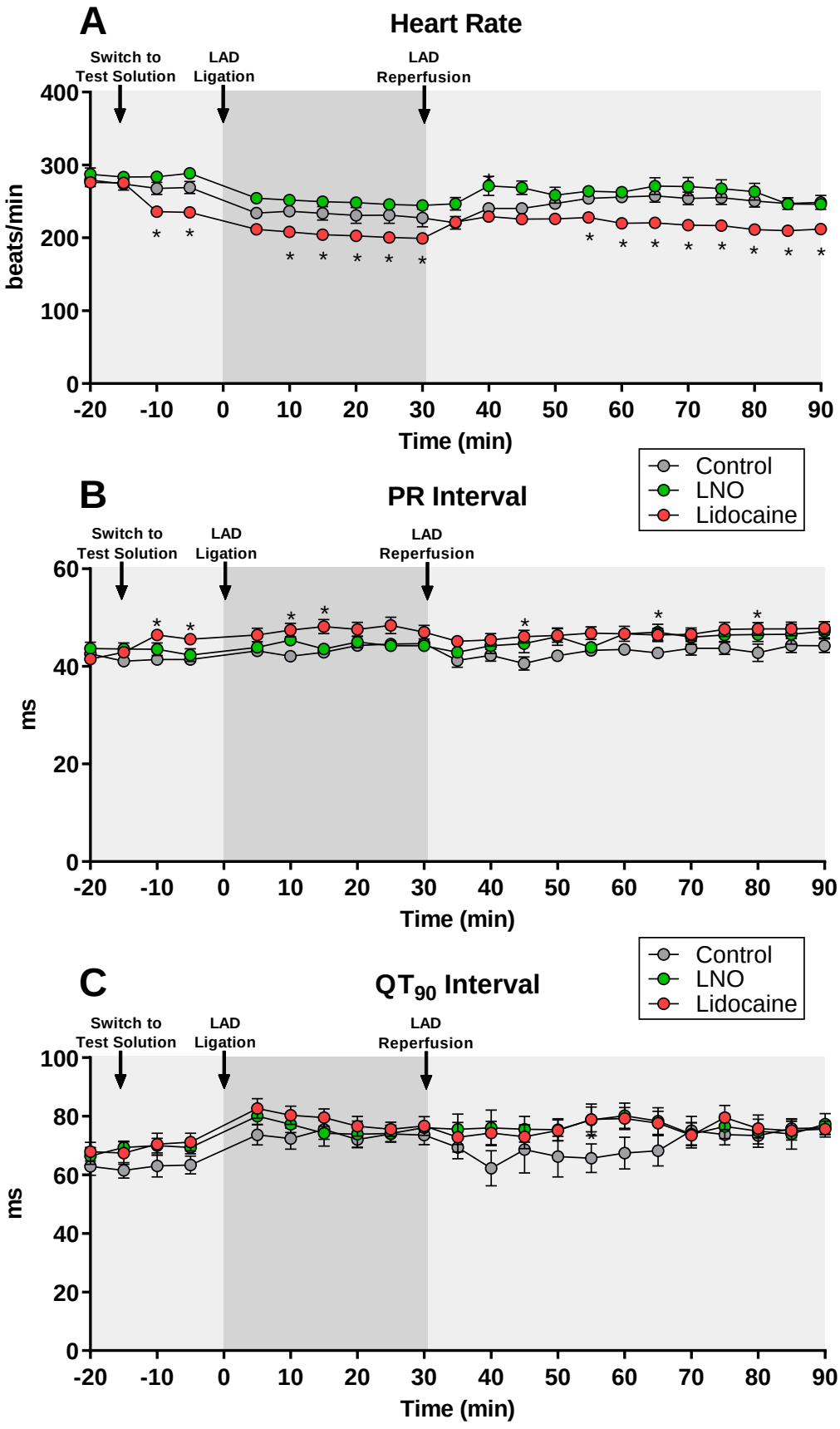


Figure 3.16 Langendorff-perfused isolated heart IVB study: Representative LV pressure and ECG traces. Representation of the differences between groups in developed pressure, heart rate and PR interval measurements taken 5 min after switch to A) Krebs control, B) 15 µM LNO, C) 15 µM lidocaine prior to onset of ischaemia. 1 box width = 250 ms. 1 box height = 5 mmHg (top 3 panels).


A
Heart Rate
Switch to Test Solution
LAD Ligation
LAD Reperfusion
beats/min
Time (min)
Control
LNO
Lidocaine
B
PR Interval
ms
C
QT90 Interval

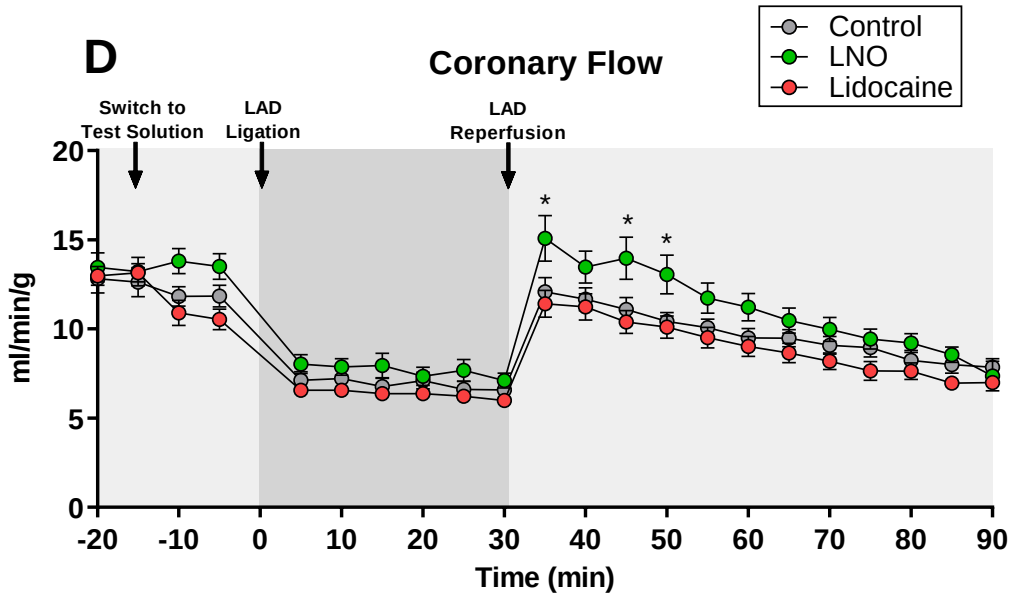


Figure 3.17 Langendorff-perfused isolated heart IVB study: Haemodynamic and ECG changes. A) Heart rate, B) PR and C) QT_{90} intervals and D) coronary flow during 30 min of regional ischaemia and 60 min of reperfusion in hearts perfused with Krebs, 15 µM LNO or 15 µM lidocaine. n=12 per group; mean ± SEM; variables were subjected to 2 way ANOVA followed by Tukey's post hoc tests (if F values were significant and data Gaussian). *p<0.05 versus control.

3.3.1.3.3 Starling Curves

There were no differences between groups for any variable during baseline Krebs perfusion (Figure 3.18A+D). Lidocaine reduced the slope of the developed pressure SC (Figure 3.18B), but had no effect on diastolic pressure (Figure 3.18E), indicating a negative inotropic effect but no lusitropic effect. LNO was without any effects (Figure 3.18B+E). Following 30 min of regional ischaemia and 60 min of reperfusion, LNO was without effect on developed or diastolic pressure SCs (Figure 3.18C). The effect of lidocaine on the developed pressure seen before ischaemia was lost, but a weak (beneficial) effect on diastolic function was observed (Figure 3.18F). Ischaemia and reperfusion caused a marked decrease in the developed pressure and increase in diastolic pressure, reflected in changes in SC slopes in all groups compared with values before ischaemia (Figure 3.18C+F).

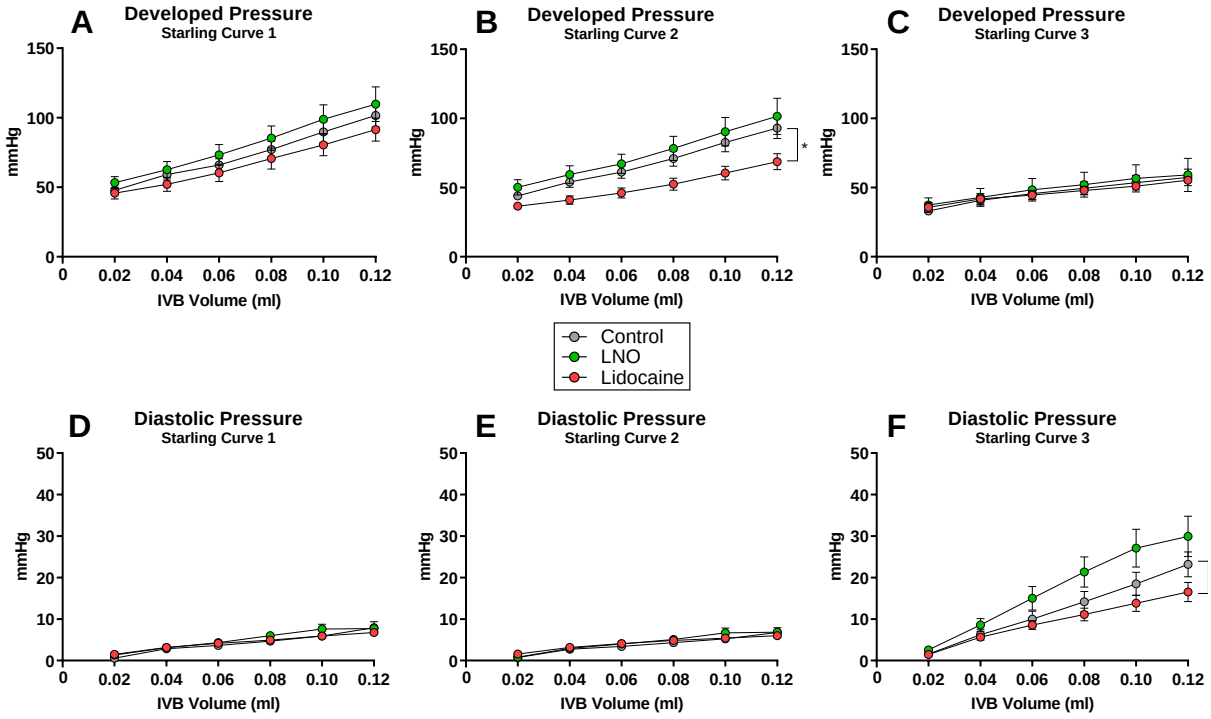


Figure 3.18 Langendorff-perfused isolated heart IVB study: SCs. A-C) Developed pressure and D-F) diastolic pressure at increasing IVB volumes in hearts perfused with Krebs, 15 µM LNO or 15 µM lidocaine. Values are from (A+D) baseline Krebs perfusion, (B+E) after switch to test solution and (C+F) at the 60th min of reperfusion after 30 min regional ischaemia. n=12 per group; mean ± SEM; *p<0.05 versus control (linear regression analysis).

3.3.2 Studies in the anaesthetised rat

3.3.2.1 Coronary ligation study

3.3.2.1.1 Arrhythmias and survival

During regional ischaemia, 100% of control rats experienced VF within 10 min of ischaemia onset (Figure 3.19A), and animal survival over 30 min was low (Figure 3.19B). Lidocaine and LNO reduced the incidence of VF (Figure 3.19A), and improved overall survival to 100% (Figure 3.19B). The antiarrhythmic effect of LNO and lidocaine was reflected by a reduced 30 min

arrhythmia score (Figure 3.19C). The outcome accords with rat Langendorff-perfused isolated heart data obtained in sections 3.3.1.1 and 3.3.1.2.

3.3.2.1.2 Haemodynamic and ECG changes

There were no differences in baseline PR and QT_{90} interval or heart rate between groups (Figure 3.19D-F). Early after ischaemia onset (e.g. 1-5 min), there were no drug-induced changes on PR or QT_{90} intervals (Figure 3.19E+F). Lidocaine caused bradycardia 1 min after ischaemia onset, but LNO did not (Figure 3.19D). Due to the high incidence of ischaemia-induced VF and early death in the control group it was not possible to analyse differences in haemodynamic and ECG variables beyond the early stages of ischaemia (Figure 3.19D-F).

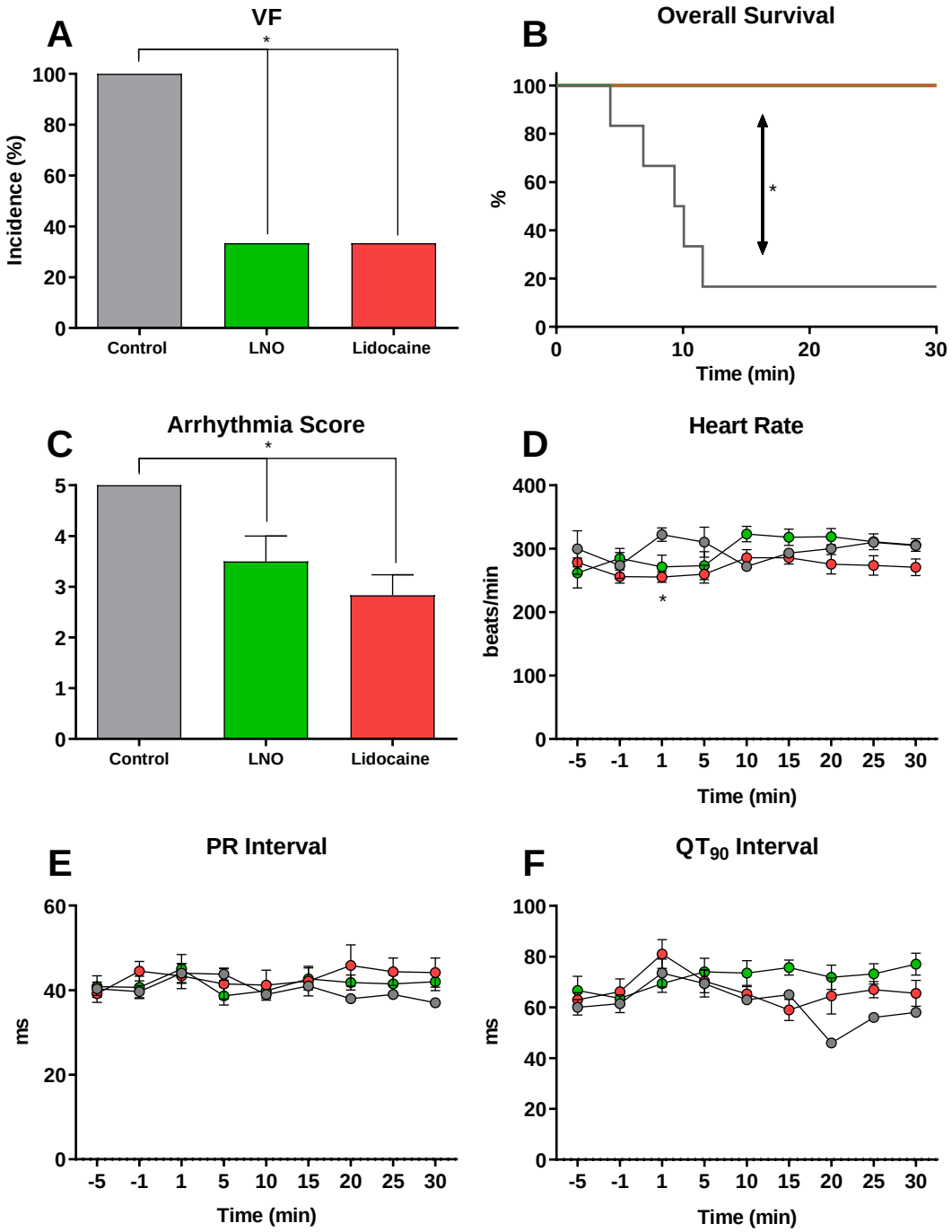


Figure 3.19 Anaesthetised rat coronary ligation study: Arrhythmias, Survival and ADRs. A) Incidence of VF, B) overall survival, C) arrhythmia score, D) heart rate, E) PR interval and F) QT_{90} interval in anaesthetised rats administered 2 mg/kg (+0.5 mg/kg/min) LNO, lidocaine, or saline (control). n=6 per group (n=1 in control group from +10 min); Binomially distributed variables (A) were compared using Fisher's exact test. Survival (B) was analysed using the log-rank (Mantel-Cox) test. Gaussian distributed variables (C-F) (mean ± SEM) were subjected to 2 way ANOVA followed by Dunnett's post hoc tests (following demonstration that F was significant and data Gaussian); *p<0.05 versus control.

3.3.2.2 Cumulative dose toxicity study

3.3.2.2.1 Haemodynamic and ECG changes

Compared with LNO, lidocaine caused bradycardia with a threshold dose of 1 mg/kg (Figure 3.21C). In addition, the PR interval was prolonged by lidocaine with a threshold dose of 32 mg/kg (Figure 3.21A). Cardiac output was lowered by lidocaine at all doses (Figure 3.21D), and stroke volume was also lowered by lidocaine at all doses greater than 2 mg/kg (Figure 3.21E). QT_{90} interval was similar in both groups at all doses (Figure 3.21B). At 64 mg/kg, lidocaine caused death in all animals from AV block and cardiovascular collapse (Figure 3.20). In contrast, LNO caused no ADRs up to 32 mg/kg (Figure 3.21A-E).

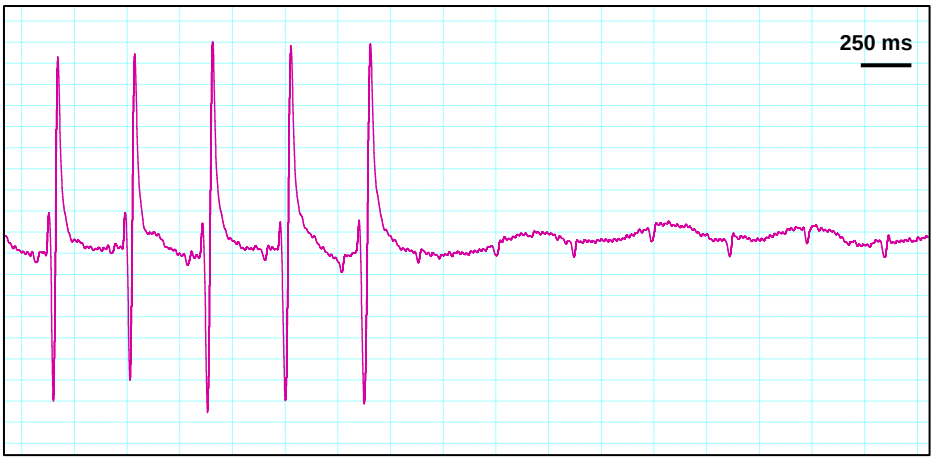


Figure 3.20 Anaesthetised rat cumulative dose toxicity study: Example of AV block observed in all animals within 2 min of administration of a 64 mg/kg bolus of lidocaine. 1 box-width = 250 ms.

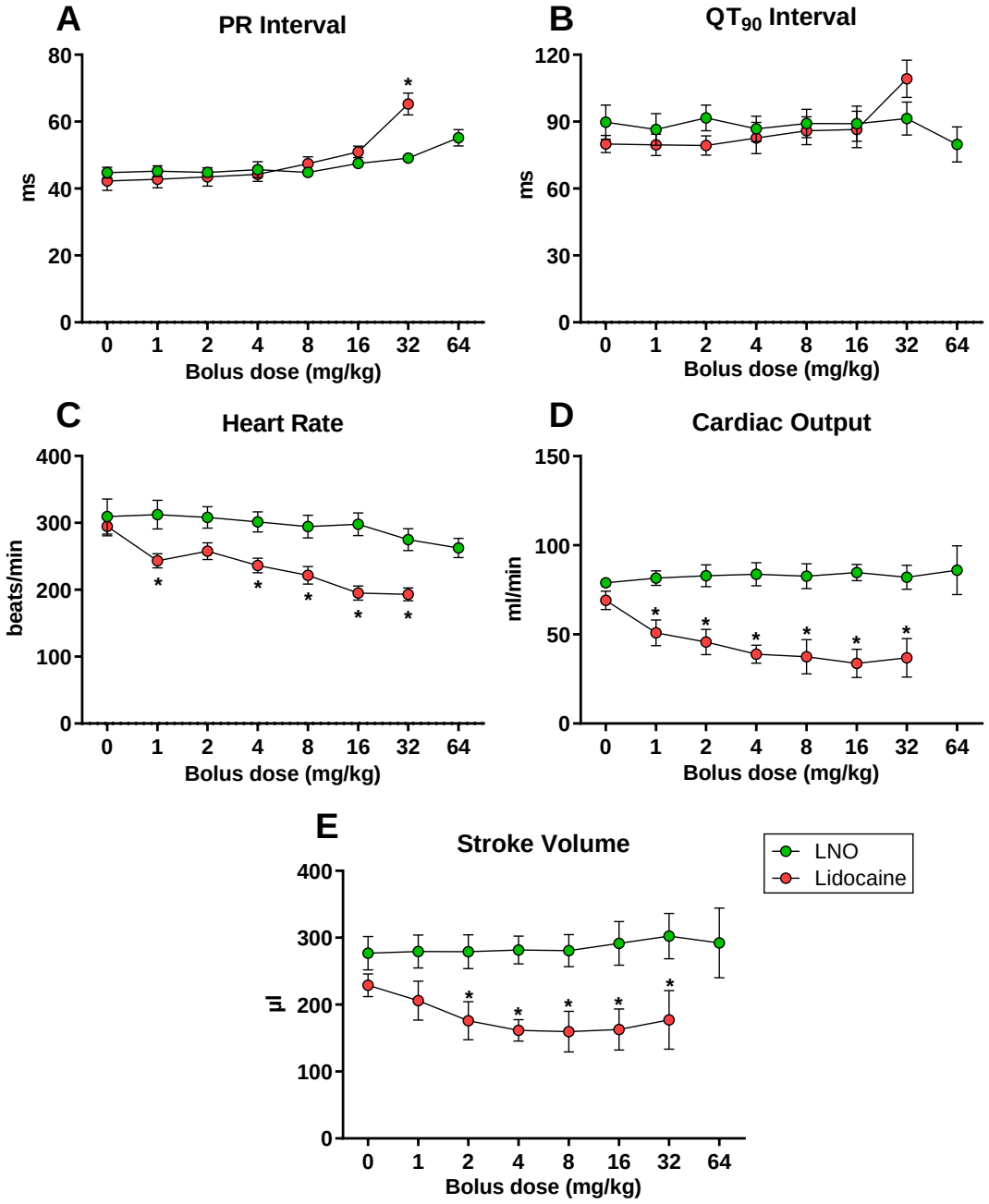


Figure 3.21 Anaesthetised rat cumulative dose toxicity study: Haemodynamic and ECG changes. A) Heart Rate, B) PR and C) QT_{90} intervals, D) Cardiac Output and E) Stroke Volume in anaesthetised rats measured approximately 1 min and 55 sec after sequential escalating drug boluses (1-64 mg/kg) given at 2 min intervals. No animal survived the 64 mg/kg lidocaine dose long enough to permit data recording. n=5 per group; mean ± SEM; Variables were subjected to 2 way ANOVA followed by Sidak's post hoc tests (if F values were significant and data Gaussian). *p<0.05.

3.4 Discussion

Initial examinations of the antiarrhythmic capabilities of LNO, in addition to its effect on ancillary variables (e.g. heart rate, PR interval, LV contractile function), were conducted in the rat Langendorff-perfused isolated heart and *in vivo* in the anaesthetised rat in order to ascertain whether LNO's TTI is superior to that of lidocaine and mexiletine.

3.4.1 Investigations into LNO's antiarrhythmic effect

3.4.1.1 Pre-ligation drug administration

In the present *ex vivo* and *in vivo* studies investigating the antiarrhythmic effectiveness of pre-ligation drug administration, LNO successfully prevented ischaemia-induced VF to the same extent as an identical concentration or dose of lidocaine. When administered before coronary occlusion, the antiarrhythmic actions of LNO and lidocaine may be accounted for by drug trapped in the LV tissue distal to the occlusion site, or present in the interface between the IZ and normal myocardium (Curtis, MacLeod and Walker, 1984), aided by additional drug accessing the IZ via collateral vessels during ischaemia (Maxwell, Hearse and Yellon, 1987). The electrophysiological antiarrhythmic mechanism of action exhibited by LNO and lidocaine in the IZ, with regards to preventing abnormal impulse formation and conduction, is the focus of chapter 4. Similarly, evidence quantifying the ischaemia-activated reduction of LNO to lidocaine in the myocardium after ligation is obtained and discussed in chapter 5. The relevant and important observation here that aids in the determination of LNO's TTI, is LNO's ability to achieve an antiarrhythmic effect equivalent to that of lidocaine.

3.4.1.2 Post-ligation drug administration

The effectiveness of LNO and lidocaine administered after the onset of ischaemia may depend on their ability to access the arrhythmogenic IZ via collateral flow (Maxwell, Hearse and Yellon, 1987; Curtis, MacLeod and Walker, 1984). Lidocaine administered i.v. commencing 2 min post-occlusion has been detected in the ischaemic LV of dog hearts after 12 min of

regional ischaemia (Weintraub, Halgash and Patterson, 1982) and, in a similar study with assessment of arrhythmias, post-ligation administration of a 40mg (~1.7 mg/kg) bolus of lidocaine was antiarrhythmic, and associated with accumulation of lidocaine (>1 μg/g) in the IZ (Davis et al., 1985). However, although collateral flow varies in dog hearts, residual flow (that remaining after coronary ligation) can be as high as 100% (complete collateralisation; Meesmann et al., 1970) so one might expect drug access to the IZ after coronary occlusion to be higher in dogs than rats, where residual flow is never more than 6% (Maxwell, Hearse and Yellon, 1987). With residual flow so low in the rat heart, this experiment to examine whether LNO and lidocaine could obtain an antiarrhythmic effect in the rat heart when delivered after coronary ligation served as a harsh proof of principle rather than any attempt to precisely recapitulate hypothetical drug dosage and administration in post-MI humans; a test of potential scope of use.

In the present studies, LNO and lidocaine both prevented ischaemia-induced VF in the rat Langendorff-perfused isolated heart when drug administration began 10 min after ischaemia onset, albeit less effectively when compared to pre-ligation drug administration (section 3.3.1.1). In anaesthetised rat hearts, the class 4 antiarrhythmic drug verapamil, when delivered after ischaemia onset, has also been shown to be able to access and accumulate in the IZ, accounting for its antiarrhythmic effect in this model, although also less effectively in comparison to when delivered pre-ligation (Curtis, MacLeod and Walker, 1984). In each case, this less potent antiarrhythmic effect compared with pre-ligation administration can be explained by the time required for drugs to access the IZ and accumulate when collateral flow is low (Maxwell, Hearse and Yellon, 1987; Curtis, MacLeod and Walker, 1984). In the case of LNO, which was less effective than lidocaine at achieving an antiarrhythmic effect when delivered post-ligation, present data would therefore imply that there is an additional step (and delay) involving its conversion to an effective antiarrhythmic concentration of lidocaine in the IZ. Furthermore, whilst collateral flow ‘tops up’ lidocaine in the IZ immediately in lidocaine-perfused hearts, lidocaine in the IZ produced from LNO in LNO-perfused hearts is instead washed out and replaced by LNO, which must then be converted to lidocaine in the IZ before enacting antiarrhythmic capabilities. These factors may explain why LNO was less

potent than lidocaine when administration began after ligation. Nevertheless, post-ligation antiarrhythmic benefit was obtained, indicating that the concept of preventing VF by administering LNO after the onset of ischaemia is viable. Analytical evidence of the accumulation and ischaemia-activated conversion of LNO administered post-ligation was not presently obtained but is referenced in chapter 5.

LNO therefore appears able to achieve its ischaemia-activated anti-VF effect irrespective of whether drug administration is commenced before or after coronary occlusion. The translation of LNO's effectiveness to humans if administration begins only after the onset of ischaemia may inevitably depend on the extent of collateral circulation in the human heart at the time of drug administration, which is variable and dependent on a variety of factors (Schaper et al., 1988). Paradoxically, the conversion of LNO to lidocaine is likely to be most swift when collateral flow is low and the inherent risk of VF consequently high. Moreover, when collateral flow is low the extent of retention of lidocaine in the IZ following the bioreduction of LNO will be greater than when collateral flow is high. This means that LNO is likely to obtain its greatest effect when the risk of ischaemia-induced VF is maximised by low collateral flow.

3.4.2 ADRs in the rat heart

The rat Langendorff-perfused isolated heart provides an early-stage approach to investigating drug effectiveness and safety in tandem, which can then be recapitulated using *in vivo* preparations. A safe antiarrhythmic drug should have disease-selectivity, for example by targeting the ischaemic territory itself, and thus be devoid of ADRs mediated outside the IZ. In the isolated heart, ADRs may be manifested as changes to heart rate, AV conduction, ventricular repolarisation or coronary flow (Hesketh et al., 2020). The class 1b drug mexiletine prolongs the PR and QT_{90} intervals in the rat Langendorff-perfused isolated heart at its threshold antiarrhythmic concentration, possessing a TTI of <2 (Hesketh et al., 2020), which fits with the drug's narrow TI in humans (Rutledge, Harris and Amsterdam, 1985). In addition, in the present studies lidocaine caused bradycardia and PR interval prolongation, as well as negative inotropy in the isolated heart and a reduction in cardiac output and stroke volume

in vivo at its antiarrhythmic concentration or dose. For a novel class 1b antiarrhythmic drug to better the 'gold standard' used against ischaemia-induced arrhythmias, it would need to display a larger TTI than mexiletine in the rat heart. Indeed, LNO was without pharmacological activity in the absence of ischaemia, at up to 4 times its antiarrhythmic concentration in the isolated heart, and at up to 30 times its antiarrhythmic dose *in vivo*. The profile of ADRs evoked by the effective antiarrhythmic concentration of lidocaine is unsurprising given the literature concerning its effect on ECG and haemodynamic variables, and the lack of ADRs exhibited by LNO is important when examining its ischaemia-activated pharmacological profile and defining its TTI.

3.4.2.1 Bradycardia and PR interval prolongation

In ischaemia studies, bradycardia commonly occurs with lidocaine in the rat isolated heart (Komai and Rusy, 1981; Farkas and Curtis, 2002) and in anaesthetised rats (Canyon and Dobson, 2004), dogs and pigs (Bergey, Nocella and McCallum, 1982). At therapeutically relevant concentrations, lidocaine causes bradycardia by reducing the rate of phase 4 diastolic depolarisation in SA nodal cells (Mandel and Bigger, 1971; Kane et al., 1983) due to blockade of I_f, leading to a decrease in SA nodal activation (Rocchetti et al., 1999). Similarly, PR prolongation, indicative of slowed AV nodal conduction (Beardow, 2000), induced by lidocaine in the present studies concurs with published studies conducted in the rat isolated heart (Komai and Rusy, 1981; Farkas and Curtis, 2002) and in the guinea pig isolated heart (Carmeliet and Zaman, 1979). The effects of lidocaine on AV conduction in the guinea pig heart are, however, less pronounced than in the rat heart, revealed only at a high drug concentration and high frequency (Carmeliet and Zaman, 1979), which is likely the result of the species differences in basal heart rate (Farmer and Levy, 1968). Lidocaine's effects on AV conduction are also less prominent than its effects on intraventricular conduction (Carmeliet and Zaman, 1979; Satoh, Narimatsu and Taira, 1981) which is the consequence of lidocaine's affinity for the *I* state of the Na^+ channel, which prevails during the AP plateau and repolarisation phase, and the longer APD in ventricular tissue (Hondeghem and Katzung, 1984).

Bradyarrhythmias caused by lidocaine have been implicated in increasing the risk of death from asystole, neutralising lidocaine's mortality benefit in humans despite its anti-VF effect (MacMahon et al., 1988; Sadowski et al., 1999). AV nodal arrhythmias and sinus bradycardia are indeed among lidocaine's recognised profile of toxic effects (Pfeifer et al., 1976). Importantly, LNO did not induce bradycardia or prolong the PR interval presently in either *ex vivo* or *in vivo* rat preparations, indicating its lack of pharmacological activity in the SA node or AV node, where ischaemia was not present. By not possessing these potentially deleterious ADRs, especially bradycardia, LNO may replicate lidocaine's benefit without mimicking its adversity.

3.4.2.2 Contractile function in the isolated heart

Lidocaine can also elicit a negative inotropic effect, seen *in vitro* (Tsuboi and Chiba, 1999; Katsuaki et al., 1996; Sheu and Lederer, 1985), *in vivo* (Wilson et al., 1993; Huang et al., 1992; Côté et al., 1973; Binnion et al., 1969), and in the clinical setting (Boudoulas et al., 1977; Bourke et al., 1987), although this is inconsistent across clinical studies (Rahimtoola et al., 1971; Matos, Hankóczy and Török, 1976; Grossman, Cooper and Frieden, 1969). These actions result from I_{Na} inhibition, which reduces intracellular Na^+ concentration, subsequently leading to decreased intracellular Ca^{2+} by way of altered Na^+/Ca^{2+} exchange and, as a result, diminished SR Ca^{2+} load and release (Sheu and Lederer, 1985; Bers, 2000; Wilson et al., 1993; Allen et al., 1983; Pankucsi, Varró and Nánási, 1996; Silva Graça and Van Zwieten, 1972; Katsuaki et al., 1996). In accordance with this, lidocaine was negatively inotropic in the present rat isolated heart study, as determined by a decreased slope of the developed pressure SC. In contrast, LNO had no effect on LV contractile function. In addition, during reperfusion after a period of regional ischaemia, neither lidocaine nor LNO had a detrimental effect on the recovery of systolic function, and lidocaine, but not LNO, appeared to improve the recovery of diastolic function.

It is possible that lidocaine's negative inotropic activity may have been underestimated due to the species-related consequences of drug-induced bradycardia in rat hearts. In the absence of sympathetic drive (the predominant influence on heart rate in rats *in vivo*), slowing the

heart rate may increase ventricular force development in the rat isolated heart, the so-called negative force-frequency relationship (FFR); this is thought to result from high SR Ca^{2+} load and high intracellular Na^{+} present at even low heart rates in the rat, limiting Ca^{2+} extrusion through the Na^{+}/Ca^{2+} exchanger (Bers, 2000). As the heart rate is increased, instead of increasing the SR Ca^{2+} load which defines the positive FFR in other species, there is less time for SR Ca^{2+} release, manifesting as negative inotropy (Bers, 2000). Elucidation of this in other species, however, is beyond the scope of this thesis, since the primary outcome from the IVB study was that LNO was without inotropic effect. Negative inotropic effects of lidocaine were observed, and the key observation is that LNO was devoid of such activity.

Although of only minor interest to the present study, it may be worth noting in passing that lidocaine-induced bradycardia and negative inotropy before and during ischaemia may also explain lidocaine's apparent benefit on the recovery of diastolic function during reperfusion. Bradycardia can provide a measure of 'anti-ischaemic' activity as manifested by a decreased ST-elevation during coronary artery occlusion (Redwood, Smith and Epstein, 1972), as well as reduced myocardial oxygen consumption (Laurent et al., 1956). In anaesthetised pigs, lidocaine transiently increased coronary blood flow and decreased myocardial function as a result of its bradycardic and negative inotropic actions, resulting in a favourable oxygen supply/demand ratio (Gee, Wilson and Angello, 1990). Similarly, in dog ischaemic myocardium, lidocaine's negative inotropy resulted in lower myocardial oxygen consumption and reduced ischaemic injury (Boudoulas et al., 1978). By the same logic, lidocaine's lack of detrimental effect on the recovery of systolic function during reperfusion may have also resulted from reduced ischaemic injury. This was not explored further as it is tangential to the main observation that LNO was not deleterious to the recovery of systolic or diastolic function during reperfusion.

3.4.2.3 Cardiac output *in vivo*

In the anaesthetised rat, lidocaine (up to 32 mg/kg) dose-dependently lowered cardiac output and stroke volume. Given lidocaine's negatively inotropic effect, as demonstrated in animal models (Tsuboi and Chiba, 1999; Katsuaki et al., 1996; Sheu and Lederer, 1985; Wilson et al.,

1993; Huang et al., 1992; Côté et al., 1973; Binnion et al., 1969) and in humans (Boudoulas et al., 1977; Bourke et al., 1987), coupled with the present results generated in the rat isolated heart, lidocaine's depression of LV contractility may contribute to the reduction in stroke volume and cardiac output secondary to bradycardia (Strubelt et al., 1971). Importantly, LNO was without effect, even up to 64 mg/kg (i.e. 32 times greater than its antiarrhythmic dose in the anaesthetised rat), and thus was pharmacologically inactive *in vivo* in the non-ischaemic rat heart.

3.4.3 The translational therapeutic index

Numerous ADRs were evaluated in the rat isolated heart simultaneously with the evaluation of beneficial effects (Hesketh et al., 2020), as discussed above, to generate a preliminary TTI (Muller and Milton, 2012) that was refined using *in vivo* data. LNO possessed a larger TTI than lidocaine, with no ADRs at up to 4-fold the antiarrhythmic concentration in the rat isolated heart. Further *in vivo* studies found that lidocaine caused bradycardia and impairment of cardiac output at a dose of 1 mg/kg, lower than its effective antiarrhythmic dose of 2 mg/kg in the same model, indicating an absence of any TTI. In contrast, LNO was without effect even at 64 mg/kg, indicating a TTI of ≥30. The 'gold-standard' class 1b drug mexiletine, had a TTI of <2 in the rat heart (Hesketh et al., 2020). LNO has a clear advantage, therefore, over current best drugs in class.

3.5 Summary

The data indicate that LNO mimics lidocaine in terms of protection against ischaemia-induced VF but without any of lidocaine's ADRs. This was predicted by, and is consistent with, the hypothesis that LNO was converted to lidocaine locally in the ischaemic milieu wherein it can exert an antiarrhythmic effect. However, mechanistic and biochemical evidence of LNO's ischaemia-activated conversion to an active antiarrhythmic agent is required to confirm this.

Chapter 4: Elucidation of the antiarrhythmic mechanism of LNO during acute ischaemia

4.1 Introduction

As a consequence of myocardial oxygen deprivation and the resulting shifts in metabolism, ionic homeostasis is disturbed during acute ischaemia, with changes culminating in an extracellular accumulation of K^+ and an intracellular accumulation of both Na^+ and Ca^{2+} (Figure 1.2) (section 1.2.1). This prompts several electrophysiological changes in the IZ, including diastolic depolarisation, APD shortening, AP V_{max} depression and a reduction in myocardial conduction velocity (Figure 1.3). These changes may lead to the flow of injury current and the precipitation of re-entrant arrhythmias (section 1.2.2). Class 1, 3 and 4 antiarrhythmic drugs are classified based upon their relative affinities for ion channels expressed in the heart, and the resulting drug-induced cardiac electrophysiological changes which confer their antiarrhythmic capabilities (Vaughan Williams, 1984; Lei et al., 2018).

The relevance of a drug's ion channel target to arrhythmogenesis, and its role in systemic ionic homeostasis, may allude to the drug's usefulness in preventing ischaemia-induced arrhythmogenesis and the likelihood that its use will be confounded by ADRs. For example, Na^+ channel inactivation early after ischaemia onset in response to diastolic depolarisation depresses I_{Na} (Carmeliet, 1999) and promotes a slowing of conduction (Kléber et al., 1986; Buchanan, Saito and Gettes, 1985) and differences in refractoriness and excitability (Shaw and Rudy, 1997a) in the IZ, leading to a period of arrhythmogenic electrical instability (Hondeghem and Cotner, 1978). Class 1 drugs i.e. I_{Na} blockers, terminate this electrical instability by prolonging tissue inexcitability and inducing bidirectional conduction block (Vaughan Williams, 1984; Lei et al., 2018), thereby reducing the risk of re-entrant arrhythmia initiation by blocking the propagation of abnormal APs (Bigger and Mandel, 1970; Hondeghem, 1987; Lazzara et al., 1978; Cardinal et al., 1981). Lidocaine's electrophysiological

properties afford some inherent tachycardia-selectivity and ischaemia-selectivity (Hondeghem and Katzung, 1977, 1984), however the extent of selectivity is evidently insufficient, resulting in concerns over ADRs. LNO's properties as a HAP depend upon the drug lacking pharmacological activity, and therefore being devoid of electrophysiological consequence, in the absence of ischaemia, becoming pharmacologically active and with antiarrhythmic effects during ischaemia (section 3.4.1) without causing ADRs in non-ischaemic supraventricular regions of the heart (section 3.4.2). Thus, LNO appears to retain the beneficial antiarrhythmic effect of lidocaine whilst possessing no action in the absence of ischaemia. This is consistent with preliminary evidence (Tien, 1999) that LNO is converted to its active drug moiety, lidocaine, selectively in the ischaemic milieu, explaining how it exerts an antiarrhythmic effect without undesired pharmacological actions and ADRs. If this is correct, the electrophysiological effects of LNO in the IZ would be expected to be identical to those of lidocaine, consistent with ischaemia-selective I_{Na} block leading to suppression of arrhythmias. Obtaining direct electrophysiological evidence of this was the focus of the present chapter.

4.2 Methods

The experiments in this chapter were completed using the methods described in section 2.1 and are summarised in Table 4.1. Specific details of the experimental protocols implemented in this chapter are provided in this section. The rats used in the ERP study, the optical mapping study and the rate-dependent conduction block study weighed 325-425 g, 320-400 g and 315-500 g, respectively. In all experiments included in this chapter, the concentrations of LNO and lidocaine used were chosen based on the data described in chapter 3. The Krebs perfusate used in the experiments in this chapter contained 4 mM KCl, rather than the 3 mM KCl used in studies in chapter 3. This alteration was made to lower the susceptibility of hearts to develop ischaemia-induced VF during LAD coronary artery ligation (Curtis and Hearse, 1989a), allowing sufficient blocs of sinus rhythm to allow determination of electrophysiological variables.

Table 4.1 Summary of studies

Study	*Details*	*Group sizes*	*Endpoint measured*
ERP study	Vehicle control	n=9	ERP at sinus rate (400 beats/min achieved by pacing)
	15 µM LNO		
	15 µM lidocaine		
Optical mapping study	Vehicle control	n=6	Conduction velocity at sinus rate (400 beats/min achieved by pacing)
	15 µM LNO		
	15 µM lidocaine		
Rate-dependent conduction block study	Vehicle control	n=9	Conduction time and conduction block at escalating pacing rates (400-900 beats/min)
	15 µM LNO		
	15 µM lidocaine		

4.2.1 ERP study

Hearts were set up for Langendorff perfusion with a LAD coronary artery ligature, ECG electrode and epicardial pacing system in place (section 2.1). The extra-stimulus pacing protocol, consisting of a series of 40 beat S1 trains (cycle length = 150 ms, 6.7 Hz) followed by a single S2 delivered at progressively shorter coupling intervals, was used to determine the ERP (ms) (from the point of loss of capture of the S2 stimulus) at five timepoints (section 2.1.6.1). After baseline Krebs perfusion, the pacing protocol was run at a constant current of 1.5 mA. Perfusate was switched to either 15 µM LNO, 15 µM lidocaine or vehicle control for 10 min prior to running the pacing protocol a second time. An IZ, anticipated to be approximately 50% TVW based on ligature positioning, was then induced by the tightening of the LAD coronary artery ligature. Three more pacing protocols were delivered at 5 min, 10 min and 20 min into ischaemia. After 20 min of ischaemia, the IZ size was delineated by the blue dye method (section 2.1.3.2) and the heart was weighed (Figure 4.1). Two min prior to the commencement of the pacing protocol, the pacing threshold was determined to

investigate changes to this parameter during drug perfusion and ischaemia, albeit all pacing protocols were conducted using the constant current of 1.5 mA.

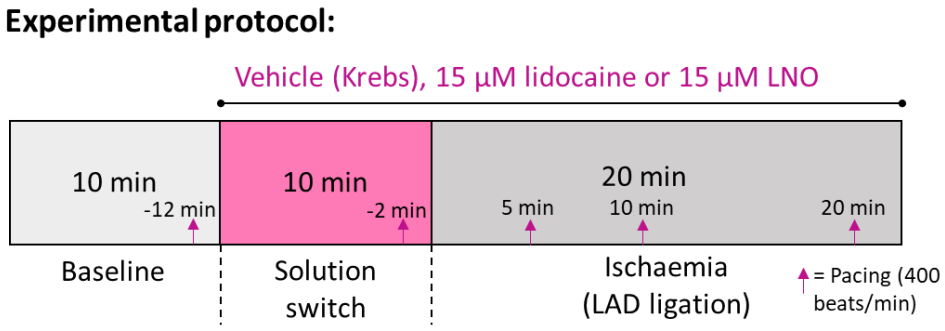


Figure 4.1 Diagram of the experimental protocol followed in the ERP study.

4.2.2 Optical mapping study

Hearts were excised, cannulated via the descending aorta and positioned for imaging of the LV (section 2.1.7), with a LAD coronary artery ligature, ECG electrode and epicardial pacing system in place (section 2.1). After baseline Krebs perfusion, the mechanical uncoupling agent blebbistatin and the voltage-sensitive dye di-4-ANEPPs were loaded into the heart (section 2.1.7) before perfusate was switched to one of three test solutions (vehicle control, 15 µM LNO and 15 µM lidocaine) and delivered for 5 min. The pacing threshold was determined, and hearts were then paced at 6.7 Hz (section 2.1.6), replicating the normal sinus heart rate of a conscious rat (approximately 400 beats/min) (Farmer and Levy, 1968; Schoemaker et al., 1990). Images were taken of the LV, and conduction velocity was calculated (section 2.1.7). Pacing was stopped whilst the LAD coronary artery was ligated, inducing an IZ anticipated to be approximately 50% of the TVW based on ligature positioning. The pacing threshold was then determined again, doubled and then pacing was resumed (6.7 Hz) to obtain a second and third series of LV images for conduction velocity analysis, run 10 min and then 20 min after the onset of ischaemia, respectively. Hearts were then removed from the apparatus and weighed (Figure 4.2).

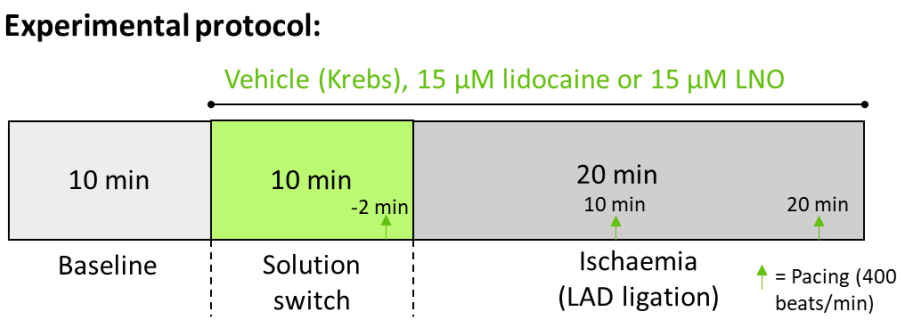


Figure 4.2 Diagram of the experimental protocol followed in the optical mapping study.

4.2.3 Rate-dependent conduction block study

Hearts were set up for Langendorff perfusion with a LAD coronary artery ligature (section 2.1) and ECG electrode and epicardial pacing system positioned as described in section 2.1.6.2. After a period of baseline Krebs perfusion, the pacing threshold of each heart was determined, doubled and then used to pace the heart at 6 pacing rates (400, 500, 600, 700, 800 and 900 beats/min) for 6 sequential 15 s periods (section 2.1.6.2). Perfusate was then switched to one of three test solutions (vehicle control, 15 µM LNO, 15 µM lidocaine) for 5 min, after which the pacing threshold of the heart was once again ascertained, doubled, and then used to run a second pacing protocol. The ligature was then tightened against the LAD coronary artery, inducing an IZ anticipated to be approximately 50% of the TVW based upon ligature positioning. The pacing threshold was determined again 5 min after the onset of ischaemia, doubled, and then used to conduct the remainder of the pacing protocols run throughout 40 min of ischaemia. Pacing protocols were run at 5, 10, 20, 30 and 40 min after ligation, after which the IZ size was delineated using the blue dye method (section 2.1.3.2). At each timepoint the pacing protocol was used to determine myocardial conduction time and the occurrence of conduction block as described in sections 2.1.6.2.1 and 2.1.6.2.2, respectively (Figure 4.3).

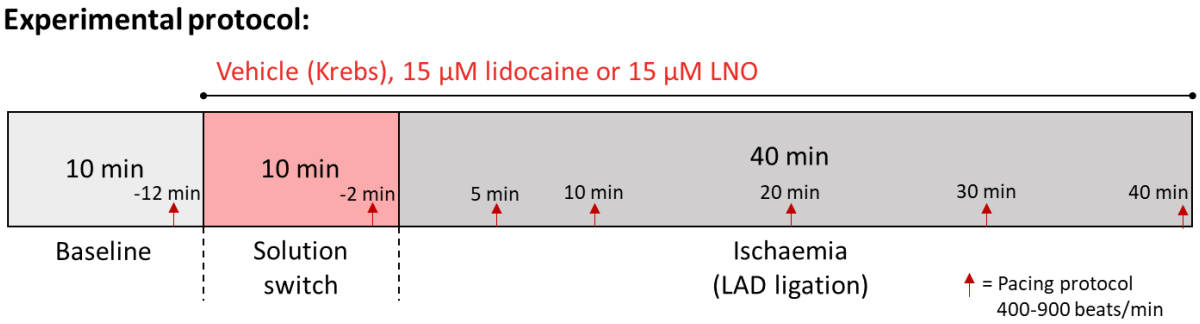


Figure 4.3 Diagram of the experimental protocol followed in the rate-dependent conduction block study.

4.3 Results

4.3.1 ERP study

4.3.1.1 Verification of experimental conditions

The mean IZ size did not differ between groups (Figure 4.4).

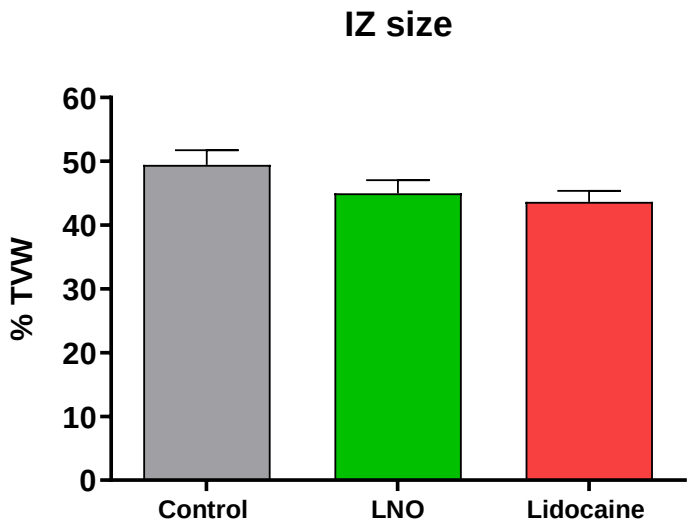


Figure 4.4 ERP study: Verification of experimental conditions. IZ size in paced (6.7 Hz) isolated hearts perfused with Krebs, 15 µM LNO or 15 µM lidocaine. n=9 per group; mean ± SEM.

4.3.1.2 ERP and pacing threshold

There was no difference in the pacing threshold in hearts paced at 400 beats/min in all groups at any timepoint, except with lidocaine at the 5th min of ischaemia, although this effect was not maintained for the duration of ischaemia (Figure 4.5B).

There were no differences between groups in the ERP measured at baseline during Krebs perfusion, or after switch to test solution perfusion (Figure 4.5A). After ischaemia onset, although ERP appears to increase slightly in the control group, this was not statistically significant (versus pre-ischaemia control values) (Figure 4.5A). The same trend was apparent in LNO-perfused hearts (Figure 4.5A). However, lidocaine caused an increase in ERP consistently throughout ischaemia versus the control group (Figure 4.5A).

It is important to note that these variables were assessed at normal sinus rhythm, whereas data from other parts of this chapter (section 4.3.3) indicate a marked rate-dependence of pharmacology of lidocaine and LNO.

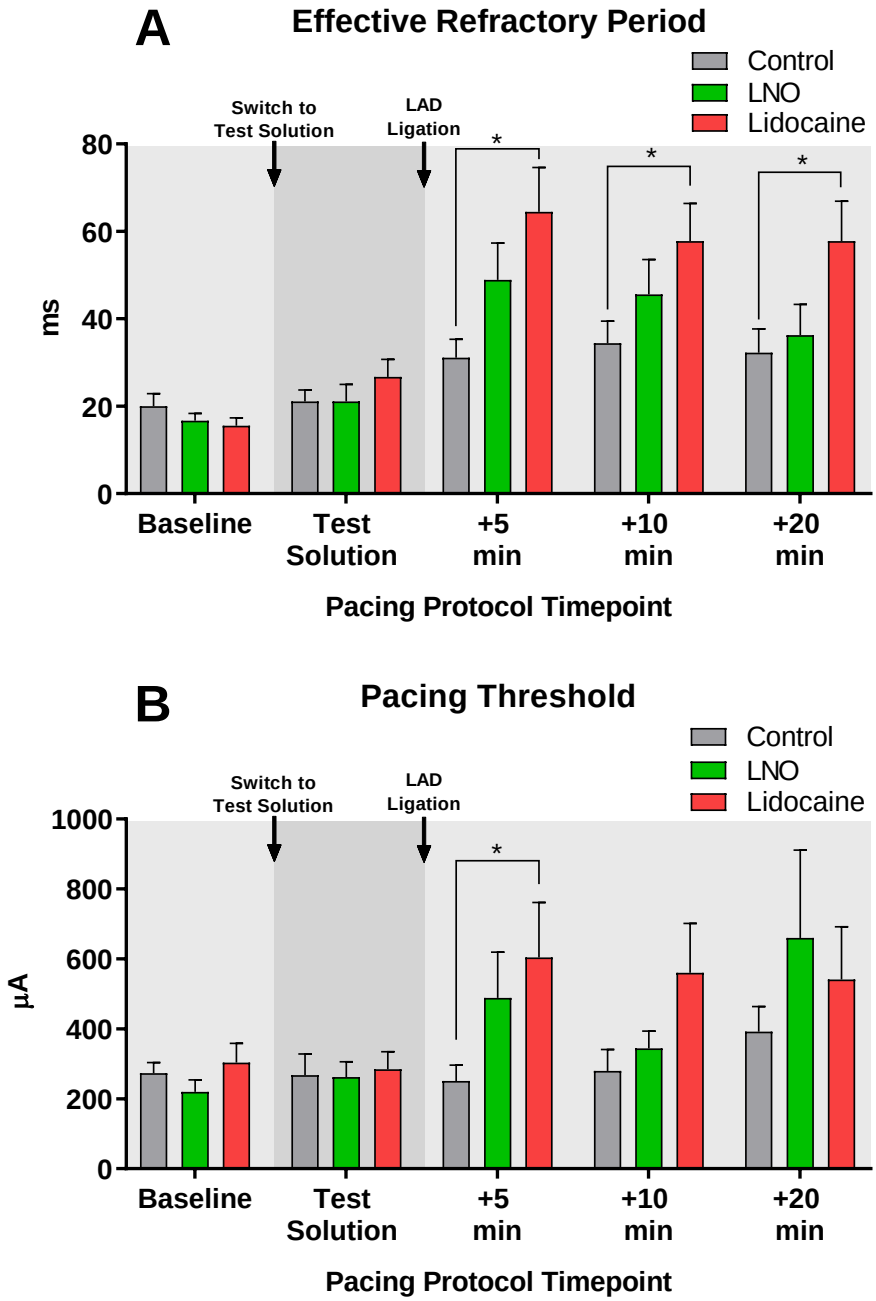


Figure 4.5 ERP study: ERP and Pacing threshold. A) ERP and B) pacing threshold determined in paced (6.7 Hz) isolated hearts perfused with Krebs, 15 µM LNO or 15 µM lidocaine during 20 min of regional ischaemia. n=9 per group; mean ± SEM; variables were subjected to 2 way ANOVA followed by Dunnett's post hoc tests (following demonstration that F was significant and data Gaussian). *p<0.05 versus control.

4.3.2 Optical mapping study

Lidocaine and LNO had no effect on LV conduction velocity in the absence of ischaemia (Figure 4.6). Ischaemia caused a reduction in LV conduction velocity in controls (Figure 4.6, Figure 4.7) and drug groups, and this effect was maintained throughout the 20 min of ischaemia (Figure 4.6). Neither lidocaine nor LNO affected LV conduction velocity during ischaemia (Figure 4.6). It is worth noting, again, that these values were determined during normal sinus rhythm.

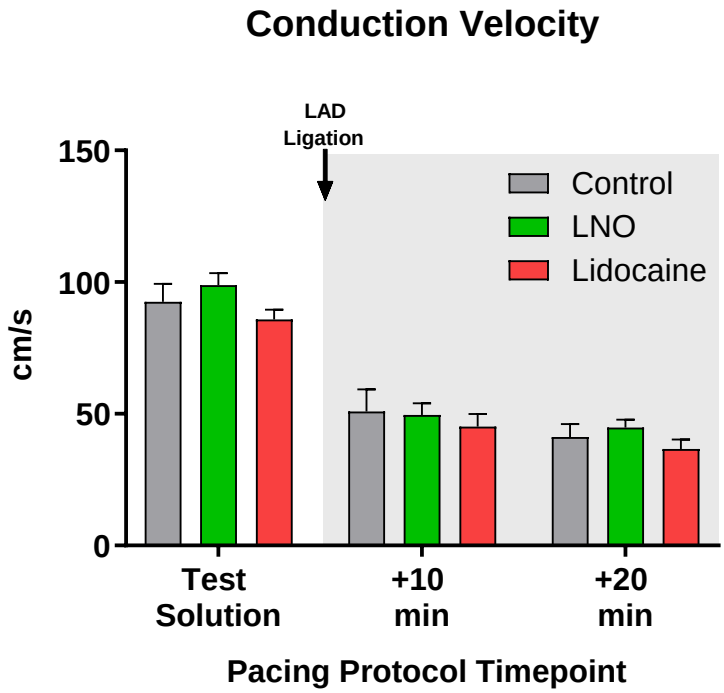


Figure 4.6 Optical mapping study: Conduction velocity. Measured by optical mapping, using di-4-ANEPPS, in paced (6.7Hz) isolated hearts perfused with Krebs, 15 µM LNO or 15 µM Lidocaine before and during 20 min of regional ischaemia. n=6 per group; mean ± SEM; 2 way ANOVA followed by Tukey's post hoc tests (following demonstration that F was significant and data Gaussian). *p<0.05 versus control.

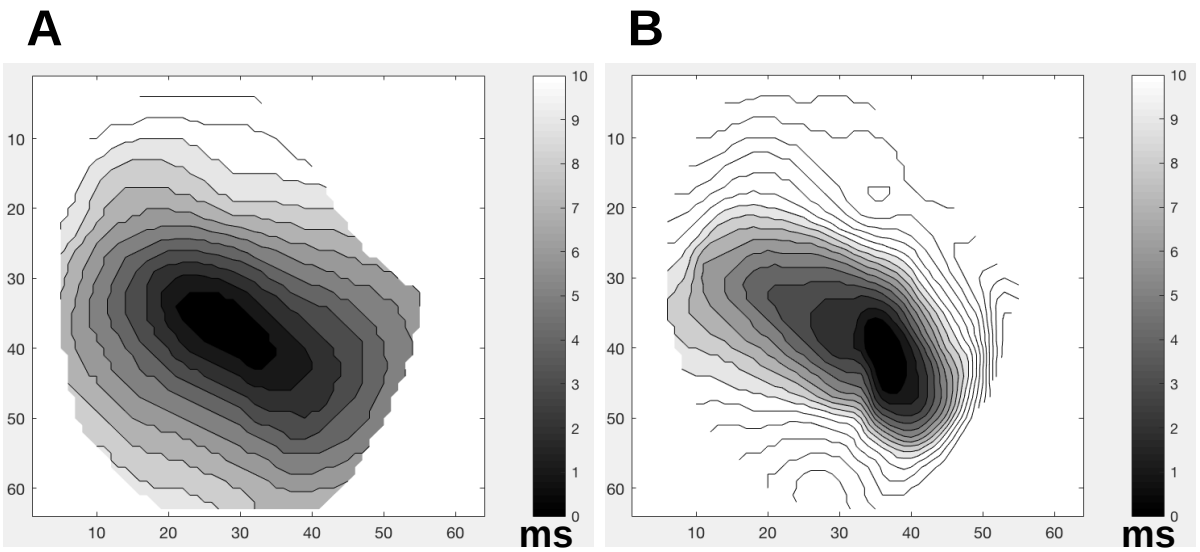


Figure 4.7 Optical mapping study: Representative optical maps. Data obtained by optical mapping, using di-4-ANEPPS, in paced (6.7 Hz) isolated hearts perfused with Krebs A) before and B) after 10 min of regional ischaemia. Data were sampled at 500 Hz with a spatial resolution of 390 µm^2 per pixel (64x64 pixels total).

4.3.3 Rate-dependent conduction block study

4.3.3.1 Verification of experimental conditions

The IZ size did not differ between groups (Figure 4.8).

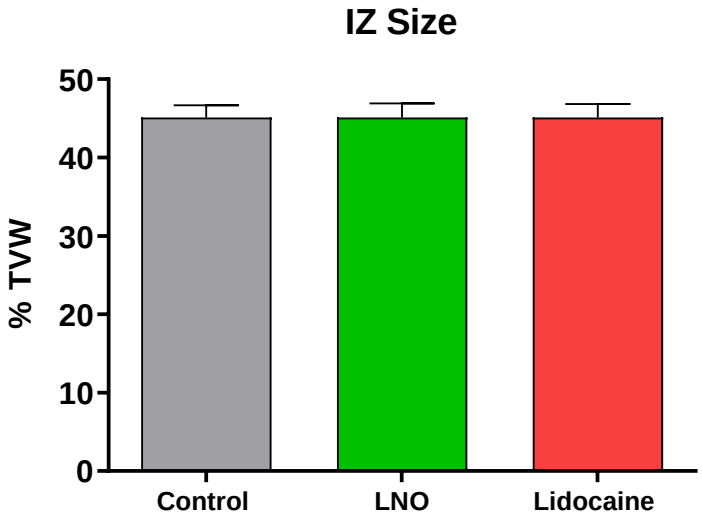


Figure 4.8 Rate-dependent conduction block study: Verification of experimental conditions. IZ size in hearts perfused with Krebs, 15 µM LNO or 15 µM lidocaine. n=9 per group; mean ± SEM.

4.3.3.2 Conduction time and conduction block

Representative conduction time measurements for each group prior to, and during, ischaemia can be found in Figure 4.9. Conduction time did not differ between groups during baseline Krebs perfusion (Figure 4.10A+B). After perfusate was switched to test solution, LNO had no effect, whereas lidocaine increased conduction time at the higher pacing rates of 600-900 beats/min (Figure 4.10A). During ischaemia, conduction slowed progressively over time in controls, and this slowing was enhanced by increasing the pacing rate (400-900 beats/min) (Figure 4.10A). By 5 min of ischaemia, existing conduction slowing was exacerbated by lidocaine and LNO to a similar degree, and rate-dependently (Figure 4.10A). By 10 min after ischaemia onset, the rate-dependence was altered such that the maximum effect was obtained at pacing rates between 400-600 beats/min, meaning conduction time was similar between the control and the drug groups at pacing rates >600 beats/min (Figure 4.10A). It was not possible to record conduction time data past 10 min ischaemia due to the high incidence of conduction block even at low pacing rates in all groups.

During baseline Krebs perfusion no conduction block was observed (Figure 4.10B). During test solution perfusion, lidocaine caused rate-dependent conduction block, with almost half of the hearts in block when paced at 900 beats/min (Figure 4.10B). This was not observed with LNO, with only one heart experiencing block in this group and only when paced at a grossly unphysiological rate of 900 beats/min (Figure 4.10B). During ischaemia, rate-dependent conduction block occurred in control hearts, and this was exacerbated by lidocaine and LNO at 5 min, with the number of hearts experiencing conduction block in the drug groups double that of controls at every pacing rate above and including 600 beats/min (Figure 4.10B). By the 10^{th} min of ischaemia, lidocaine and LNO caused conduction block in most hearts at low pacing rates (400-600 beats/min), in contrast to controls (Figure 4.10B). After 20 min of ischaemia, conduction block occurred at lower pacing rates in all groups with drug effects now lost (Figure 4.10B).

Thus, whilst the effect of the drugs on conduction time was most evident 5 min after the onset of ischaemia, rate-dependent conduction block in the IZ was most evident 10 min after the

start of ischaemia. LNO had no effect on conduction time and did not cause conduction block until and unless ischaemia was present, in which case its effects were identical to those of lidocaine.

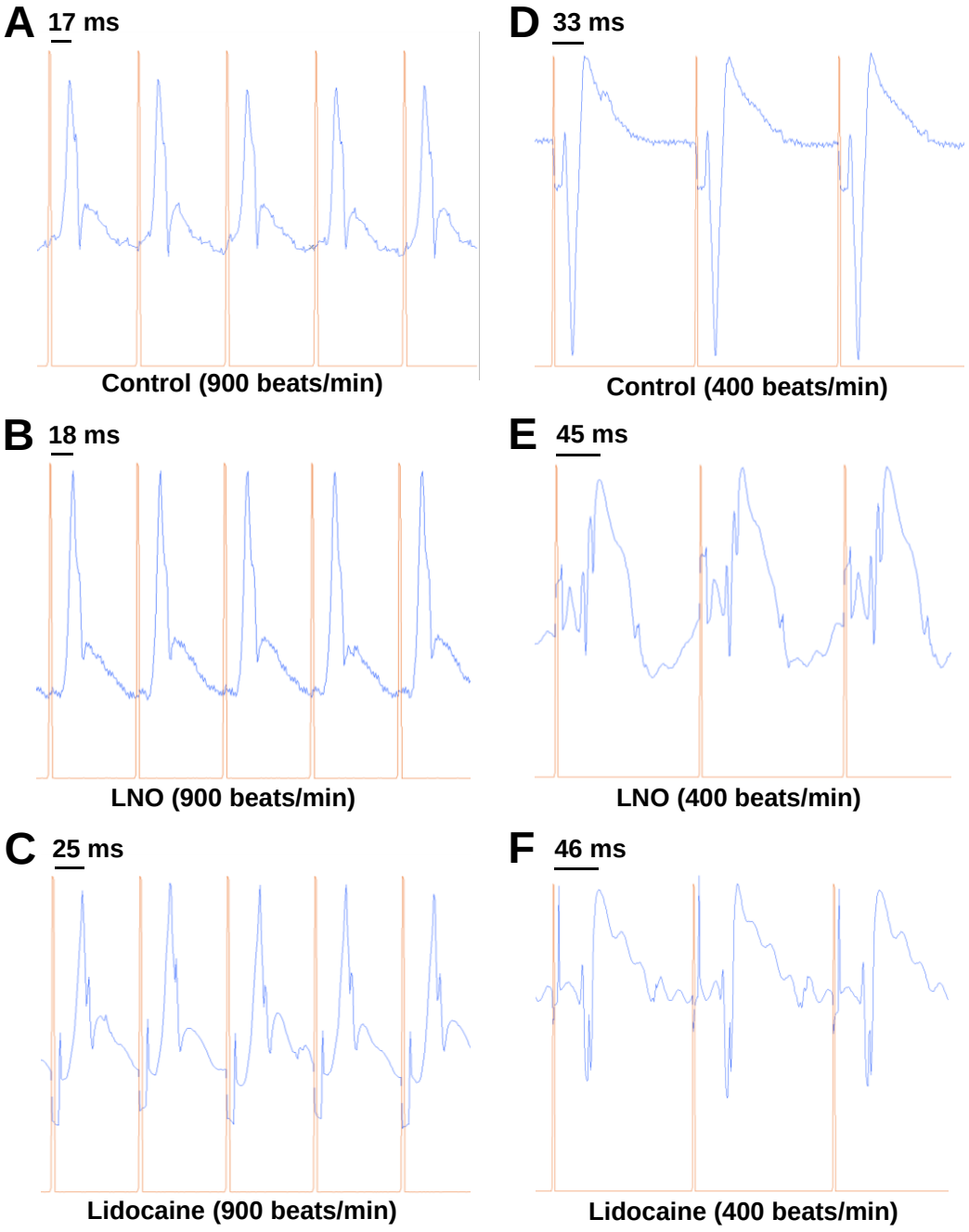


Figure 4.9 Rate-dependent conduction block study: Representative conduction time measurements. Examples of conduction time ('stimulus to QRS peak interval in ms) in isolated hearts perfused with Krebs, 15 µM LNO or 15 µM lidocaine A-C) after switch to test solution (pacing at 900 beats/min to display cumulative rate-dependent effects in absence of ischaemia) and D-F) 10 min after ischaemia onset (pacing at 400 beats/min to display exacerbation of ischaemia-induced conduction slowing).

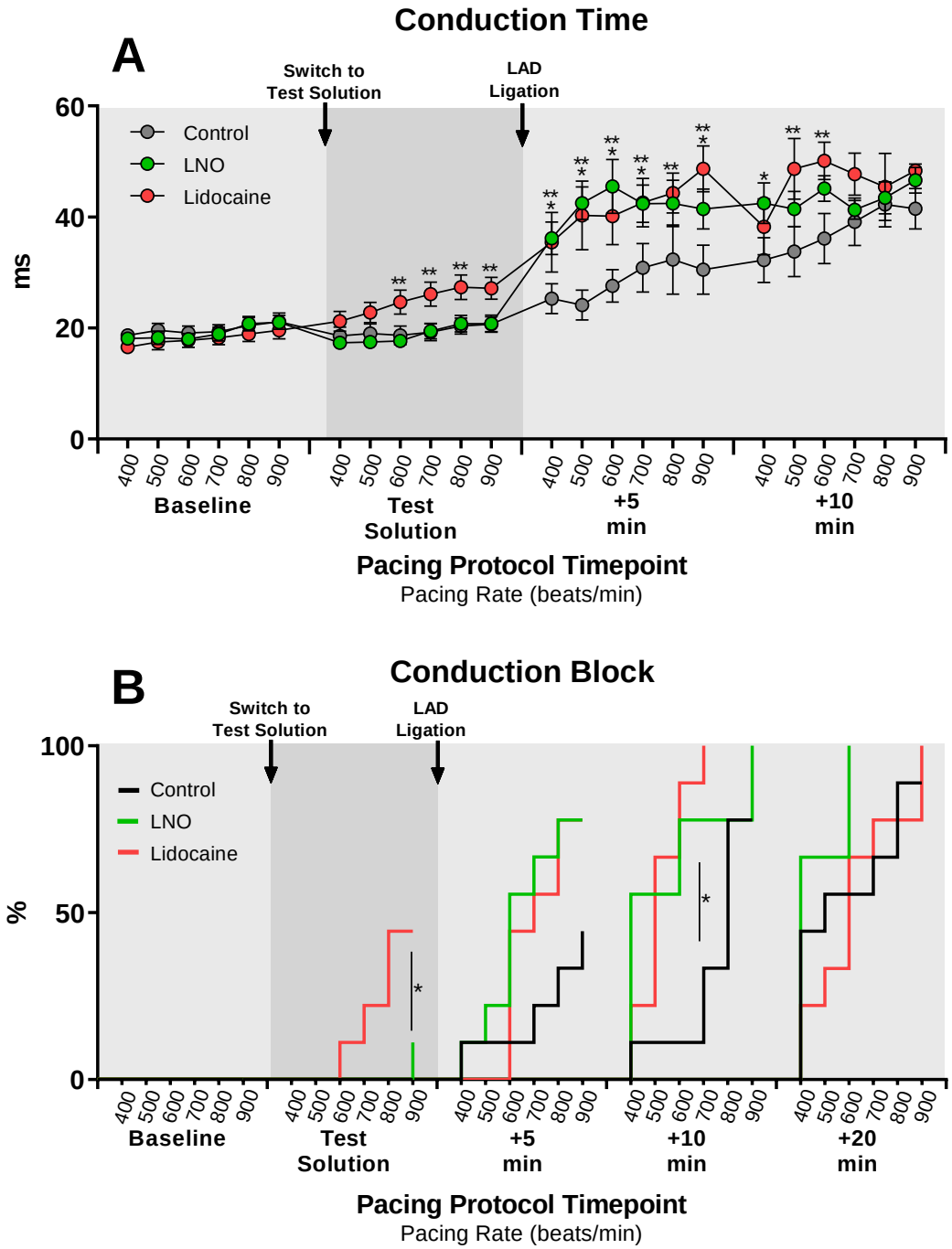


Figure 4.10 Rate-dependent conduction block study: Conduction time and block. A) Conduction time and B) occurrence of conduction block in paced (400-900 beats/min) isolated hearts perfused with Krebs, 15 µM LNO or 15 µM lidocaine during 40 min of regional ischaemia (results from up to 20 min ischaemia displayed). n=9 per group; Conduction time (A) (mean ± SEM) was evaluated by 2 way ANOVA followed by Tukey's post hoc tests (as F was significant and data Gaussian). Conduction block (B) (percentage) was analysed using the log-rank (Mantel-Cox) test. *p<0.05 versus control.

4.4 Discussion

4.4.1 The antiarrhythmic mechanism of I_{Na} block

The characteristics of I_{Na} are altered during acute ischaemia, driving arrhythmogenesis (section 1.2.1). Re-entry is the principal cause of arrhythmogenesis during acute ischaemia and is characterised by a continuous loop of abnormal conduction which, after each loop, generates the distal propagation of a tachycardic impulse (section 1.2.2.1). Provided the head of a re-entrant wavefront encounters excitable tissue throughout the pathway's entirety, and its conduction time is greater than its refractory time, the circuit will continue, only ceasing if the electrical characteristics of the circuit change (Antzelevitch and Burashnikov, 2011; Gaztanaga, Marchlinski and Betensky, 2012; Tse, 2016). Whilst ischaemia-induced VT results from the formation of these large re-entrant circuits in the IZ, their fragmentation into multiple wavelets is the likely cause of the degeneration of VT into VF (Janse et al., 1980). Regionally slowed conduction velocity during myocardial ischaemia, caused by reduced Na^+ channel availability resulting from diastolic depolarisation-induced inhibition of I_{Na} (Kléber et al., 1986; Kléber and Rudy, 2004) in addition to ischaemia-induced extracellular K^+ accumulation (Coronel, 1994) and the resultant changes to I_{Na} leading to heterogeneities in excitability and refractoriness (Kléber and Rudy, 2004) increase the likelihood of the flow of injury current and unidirectional block developing, ultimately increasing re-entry risk (section 1.2.2.1). In this regard, the diminishing availability of I_{Na} during acute ischaemia is a major determinant of the initiation of arrhythmogenesis, particularly re-entry.

Class 1b antiarrhythmic drugs prevent ischaemia-induced arrhythmogenesis by converting unidirectional block and slowed regional conduction velocity into bidirectional conduction block by prolonging the ERP beyond the point of repolarisation (Vaughan Williams, 1984; Lei et al., 2018), thereby preventing premature abnormal AP propagation and decreasing the likelihood of re-entrant circuit formation and maintenance (Bigger and Mandel, 1970). The risk of re-entry is greatest during a vulnerable window of markedly slowed (but not blocked) conduction resulting from the reduced availability of openable Na^+ channels (Kléber and Rudy, 2004; Hondeghem and Cotner, 1978). As ischaemia progresses, openable Na^+ channels

diminish further owing to diastolic depolarisation-induced inactivation, and conduction changes from slowed to blocked (Hondeghem and Cotner, 1978). This coincides with, and explains, the hiatus in arrhythmia susceptibility that manifests in hearts of animals surviving the early stages of ischaemia (Curtis, 1998). The extent of Na^+ channel block and inactivation therefore determine arrhythmia susceptibility, and class 1b I_{Na} blockers hasten the time to bidirectional conduction block and electrical quiescence during ischaemia, consequently reducing the time spent in the window of arrhythmia vulnerability (Kléber and Rudy, 2004; Hondeghem and Cotner, 1978; Hondeghem, 1987).

4.4.2 The antiarrhythmic mechanism of LNO

The antiarrhythmic mechanism of LNO was systematically examined from key aspects of its antiarrhythmic electrophysiological effects during ischaemia compared with those of lidocaine, a drug well-characterised (Vaughan Williams, 1984; Lei et al., 2018), with the expectation that LNO would mimic lidocaine's effects, all referable to I_{Na} block (i.e. ERP prolongation and bidirectional conduction block), with the difference between the actions of lidocaine and LNO being solely the absolute dependency on the presence of ischaemia in the case of LNO.

4.4.2.1 Prolonging the ERP

There is a close temporal relationship between APD and the ERP in non-ischaemic tissue, but this is disturbed by myocardial ischaemia (Sutton et al., 2000; Burton and Cobbe, 2001). APD shortens at the same time changes to resting membrane potential, AP amplitude and V_{max} occur (Zhang et al., 2005) and yet the ERP may prolong (Janse et al., 1985) or shorten (Zhang et al., 2005), and this may lead to differences in excitability and refractoriness in different regions of the myocardium (Shaw and Rudy, 1997a). Drug-induced blockade of I_{Na} during ischaemia may cause slow recovery from the *I* state which prolongs the ERP beyond the point of repolarisation, blocking premature stimuli and the propagation of re-entrant impulses (Bigger and Mandel, 1970). In the present study of the ERP in the rat Langendorff-perfused isolated heart paced at sinus rate, the onset of ischaemia caused a prolongation of the ERP,

and this plateaued as ischaemia progressed, a trend which has been observed previously (Opthof et al., 1993). Lidocaine had no effect on the ERP in the absence of ischaemia, in line with published observations (Kupersmith, 1979), and nor did LNO. However, during normal sinus rhythm lidocaine prolonged the ERP early after ischaemia onset, an effect that was maintained during ischaemia and anticipated based on previous studies (Kupersmith, 1979; Wolk et al., 1998). LNO had no significant effect.

The effect of lidocaine on the ERP is well-described and attributable to I_{Na} block in the IZ (Lei et al., 2018; Vaughan Williams, 1984; Bigger and Mandel, 1970). The effects during normal sinus rhythm observed here were weak, and the absence of such effects with LNO was unsurprising given the conversion of LNO to lidocaine during ischaemia may not follow a 1:1 stoichiometry (Tien, 1999), which is observed and discussed subsequently in chapter 5. Thus, although the outcome of this experiment may appear disappointing, it is not of wider concern since LNO is not required to affect electrophysiology during normal sinus rhythm to suppress VF. Therefore, the 'trend' of an effect of LNO in the study was not of sufficient relevance to warrant increasing group sizes to seek whether it was a 'real' effect. Despite LNO's minimal effect on the ERP during normal sinus rhythm, the drug still achieves an antiarrhythmic effect against ischaemia-induced VF (section 3.3).

4.4.2.2 Reducing conduction velocity in the IZ

I_{Na} block can have arrhythmogenic consequences if conduction is slowed rather than blocked (Ranger et al., 1989). Lidocaine's effect on myocardial conduction velocity is limited to the IZ, and is exacerbated during tachycardias (Davis et al., 1986; Hondeghem and Katzung, 1977, 1984; Hondeghem, 1987). Therefore, to establish whether LNO exhibited effects on conduction indicative of I_{Na} block in the IZ also demonstrated by lidocaine (Bigger and Mandel, 1970; Cardinal et al., 1981; Lazzara et al., 1978) the effects of both agents on myocardial conduction velocity during ischaemia at sinus rate was investigated in the rat Langendorff-perfused isolated heart using optical mapping techniques (section 4.3.2). In line with previous studies (Davis et al., 1986; Morady et al., 1985; Okumura et al., 1988), lidocaine did not decrease conduction velocity at sinus rate in the absence of ischaemia, and neither did LNO.

The onset of ischaemia caused conduction velocity to rapidly and substantially slow (>50% pre-ischaemia values), as expected (Kléber et al., 1986; Buchanan, Saito and Gettes, 1985), but this effect was not potentiated by either lidocaine or LNO. The implication is that lidocaine achieved insufficient I_{Na} block at sinus rate to adversely affect ischaemia-induced conduction slowing, consistent with the findings of others (Davis et al., 1986; Carson and Dresel, 1983). Importantly, LNO was also without effect.

Given the lack of effect of LNO, and weak effects of lidocaine, on the ERP and conduction velocity during ischaemia at normal sinus rhythm, this prompted an investigation into the rate-dependence of the effects of LNO during ischaemia.

4.4.2.3 Hastening the time to conduction block rate-dependently

The electrophysiological effect of lidocaine's I_{Na} block during ischaemia is known to be exacerbated during tachycardias (Davis et al., 1986; Hondeghem and Katzung, 1977), and at antiarrhythmic concentrations is manifested as the production of bidirectional conduction block (Cardinal et al., 1981; Lazzara et al., 1978). In view of this, and the new conduction data discussed above, it was examined whether the marked antiarrhythmic effects of LNO are associated with an ischaemia-dependent tachycardia-selective pharmacology. To this end, the rat Langendorff-perfused isolated heart was paced at a range of frequencies, before and during regional ischaemia, and endpoints indicative of I_{Na} block were measured, namely myocardial conduction time and the occurrence of conduction block (Cascio et al., 1987).

In the absence of ischaemia, a slight rate-dependent slowing of conduction occurred in control hearts between 400 and 900 beats/min (by approximately 5 ms). The pattern was identical in the LNO group. In contrast, lidocaine exacerbated rate-dependent conduction slowing over the range 600-900 beats/min. In addition, whilst conduction block was absent in non-ischaemic control hearts, lidocaine evoked rate-dependent conduction block in 45% of hearts in the absence of ischaemia. Taken together, these observations are evidence of lidocaine-induced ischaemia-independent tachycardia-selective I_{Na} block, consistent with beat-to-beat cumulative I_{Na} block manifesting during high heart rates equivalent to those seen during tachyarrhythmias in rats (e.g. >400 beats/min (Schoemaker et al., 1990; Farmer and

Levy, 1968)). LNO had no such activity. In the absence of ischaemia, however, one heart developed conduction block at 900 beats/min in the presence of LNO. It is noteworthy that LNO had no commensurate effect on conduction time before ischaemia. Therefore, the one instance of conduction block is likely a chance event. If real, it may be the case that the pathologically high pacing rate (Ferdinandy, Das and Tosaki, 1993) may have caused pacing-induced myocardial ischaemia (Takeda et al., 1995) sufficient to convert some LNO to lidocaine. Nevertheless, LNO clearly did not mimic the I_{Na} blocking pharmacological actions of lidocaine in the absence of ischaemia.

The modest rate-dependent increase in conduction time present at baseline in control hearts was increased by ischaemia after 5 min. This occurred to a similar extent to that caused by lidocaine prior to ischaemia. These effects are expected and typical of reduced I_{Na} availability (Kléber et al., 1986; Buchanan, Saito and Gettes, 1985). In noting that the rat Langendorff-perfused isolated heart rarely exhibits arrhythmias during the first 5 min of regional ischaemia (Curtis, 1998), the degree of slowed conduction caused by lidocaine before ischaemia would not be expected to carry a risk of proarrhythmia. There was a marked increase in conduction time 5 min after ischaemia onset caused by lidocaine versus controls, as was expected from published studies (Hope et al., 1974; Davis et al., 1986; Carson and Dresel, 1983). Importantly, LNO evoked effects on conduction time almost identical to those of lidocaine at that time point. In addition, after 5 min of ischaemia there was a pattern of conduction block that matched the pattern of conduction slowing, with conduction block approximately twice as likely in the two drug groups versus controls over the pacing range 600-900 beats/min. After 10 min of ischaemia, the extent of conduction slowing in the lidocaine and LNO groups was not further increased, and the rate-dependence of both drugs saturated at lower rates, indicative of increasing sensitivity to tachycardia. In addition, although the conduction slowing effects of lidocaine and LNO reached their maximum after 5 min ischaemia, conduction block with LNO and lidocaine became most prevalent at lower heart rates 10 min after ischaemia onset, at which point there is a clear distinction between conduction block obtained in hearts with lidocaine and LNO at relatively low pacing rates, and that occurring predominantly at higher pacing rates in control hearts. Notably, the risk of ischaemia-induced VF increases markedly from approximately 10 min after ischaemia onset in this model, with

peak susceptibility occurring at approximately 10-20 min (Curtis, 1998). Rate-dependent conduction block increased in severity in all groups as ischaemia progressed, and by 20 min the extent of conduction block was similar in all groups, at which point the severity of ischaemia-induced arrhythmias arising in this model is diminished (Curtis, 1998) and ischaemic tissue begins to reach electrical quiescence (Hondeghem and Cotner, 1978). This indicates that the rate-dependence of conduction slowing and block produced by lidocaine and LNO is shifted by ischaemia, consistent with a synergistic relationship between drug and ischaemia on Na^+ channel availability, ultimately hastening the transition between ischaemia-induced conduction slowing and rate-dependent bidirectional conduction block (Hondeghem and Cotner, 1978; Hondeghem and Katzung, 1977). Facilitation of lidocaine's rate-dependent conduction block by ischaemia is a well-described process (Harper et al., 1993; Hondeghem and Cotner, 1978; Hope et al., 1974; Davis et al., 1986; Carson and Dresel, 1983). The novel and interesting observation was that LNO mimicked lidocaine in all respects, except that LNO had no pharmacological activity prior to ischaemia. One may therefore describe LNO's actions as ischaemia-dependent and tachycardia-selective, whereas lidocaine's almost identical pharmacology is ischaemia-selective and tachycardia-selective. Ischaemia-dependence is superior to ischaemia-selectivity in that effects are present only under ischaemic conditions. The term ischaemia-selective refers to relative effects in ischaemic and non-ischaemic tissue which favour ischaemic tissue, but, in the case of lidocaine, are not disparate enough to prevent ADRs and rate-dependent changes to conduction.

It is worthwhile to note that the suppression of ischaemia-induced VF by LNO and lidocaine is not attributable to drug-induced conduction slowing, which is proarrhythmic (Antzelevitch and Burashnikov, 2011; Gaztanaga, Marchlinski and Betensky, 2012; Tse, 2016). Conduction slowing is instead an unavoidable prelude to bidirectional conduction block (Vaughan Williams, 1984; Lei et al., 2018; Cardinal et al., 1981; Lazzara et al., 1978), and the suppression of VF by LNO and lidocaine is attributable to a reduction in the duration of arrhythmogenic conduction slowing (Hondeghem and Cotner, 1978; Hondeghem, 1987) as discussed above.

4.5 Summary

Lidocaine and LNO produce rate-dependent bidirectional conduction block in the IZ, explicable by the potentiation of ischaemia-induced Na^+ channel inactivation, sufficient to account for suppression of ischaemia-induced VF. The drugs differ only in terms of LNO's absolute dependency on the presence of ischaemia to exert an effect. These observations add to the accumulating evidence that LNO is an ischaemia-activated tachycardia-selective antiarrhythmic drug with no pharmacology in the absence of ischaemia.

Chapter 5: Quantification of the reduction of LNO to lidocaine in the ischaemic myocardium

5.1 Introduction

Despite its evident dependence on the presence of ischaemia to exert its pharmacological actions, the direct evidence that LNO is converted in ischaemic heart tissue to lidocaine is limited to data in a 22 year old PhD thesis (Tien, 1999). The present chapter addresses this. Studies to investigate the hypoxia-activated metabolism of other HAPs have been conducted using HPLC with MS/MS analysis. In one study, the analysis of solid tumour and normal tissue biopsied from patients treated with AQ4N, an inert di-N-oxide prodrug of the cytotoxic agent AQ4 (section 1.4.1.1), revealed that, whilst AQ4N was detectable to a larger degree in normally perfused tissue adjacent to cancerous tissue, AQ4 was detected almost exclusively in hypoxic tissues (Albertella et al., 2008). This suggested that AQ4N was capable of penetrating poorly perfused hypoxic tumour tissue, wherein it was bioreduced to AQ4, with the potential to improve disease outcomes without adverse drug toxicity (Albertella et al., 2008). HPLC-MS/MS methodology validated in rat, mouse and human tissues has also been developed to aid biodistribution analysis of PR-104A and its HAP, PR-104, in clinical trials (Patel et al., 2007).

In this chapter, the presence of LNO and lidocaine in samples of rat heart tissue exposed to ischaemic or non-ischaemic conditions was examined using a systematically determined experimental protocol and a newly established UHPLC-MS/MS analysis method.

5.2 General methods

The experiments in this chapter were completed using the methods described in sections 2.1 and 2.3 and are summarised in Table 5.1. Samples of myocardial tissue were collected from rat Langendorff-perfused isolated hearts subjected to three different experimental protocols

Chapter 5: Quantification of the reduction of LNO to lidocaine in the ischaemic myocardium

(Table 5.1). Emerging results informed alterations to the first protocol and so on. Rats weighing 310-400 g were used in the experiments in this chapter.

Table 5.1 Summary of studies

Study	*Details*	*Duration and type of ischaemia*	*Freezing method*	*H_2O_2 concentration added*
First protocol	15 µM LNO	30 min regional ischaemia	Tissue cut during perfusion then frozen in liquid nitrogen	N/A
Second protocol	15 µM LNO 15 µM lidocaine	30 min global ischaemia	Snap frozen with cryogenic tongs	2%
Third protocol	15 µM LNO 15 µM lidocaine	10 min global ischaemia	Snap frozen with cryogenic tongs	1%

5.3 First protocol

5.3.1 Methods

Wistar rat (310-380 g) hearts were set up for Langendorff perfusion, as in section 2.1. Following 5 min of Krebs perfusion, the perfusate was switched to 15 µM LNO for a period of 10 min. Hearts were then randomised to undergo either a period of 30 min of regional ischaemia by means of tightening an LAD coronary artery ligature (producing a region of ischaemia anticipated to be 50% of the TVW) (section 2.1.3) (n=6) or a period of time-matched normal perfusion (normoxia) with no ligature (n=6). At the 30th min of ischaemia, or time-matched normoxia, samples of ventricular tissue weighing approximately 30 mg were dissected from the LV, during continued perfusion, using forceps and scissors. Samples were weighed and stored in liquid nitrogen in CryoTube Vials (Thermo Scientific, US) (Figure 5.1). Test samples and 9 calibration standard samples were then subsequently prepared for UHPLC-MS/MS experimentation as described in section 2.3. No H_2O_2 was added during the sample preparation for UHPLC-MS/MS analysis as part of this first protocol (section 2.3.3).

The 30 min duration of regional ischaemia was chosen to mimic the conditions under which the antiarrhythmic effectiveness of 15 µM LNO had been established (section 3.2.1.1).

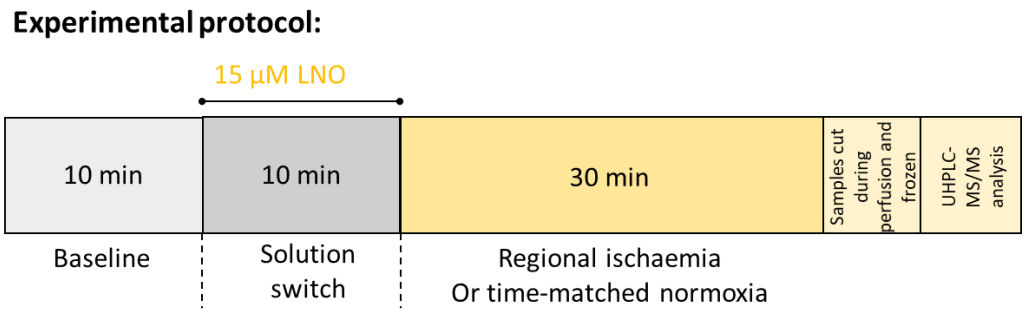


Figure 5.1 Diagram of the first experimental protocol followed to detect the presence of LNO and lidocaine in normoxic and ischaemic myocardial tissue from rat Langendorff-perfused isolated hearts.

5.3.2 Results

Standard calibration curves were constructed for lidocaine and for LNO on the same day that test samples were analysed. Detection was linear over the range 0-250 ng/ml, and the coefficient of determination (r^2) was 0.9999 for LNO (Figure 5.2A) and 0.9999 for lidocaine (Figure 5.2B). The correlation equations were y = 0.00125x - 0.0005339 and y = 0.01347x - 0.001673 for LNO (Figure 5.2A) and lidocaine (Figure 5.2B), respectively.

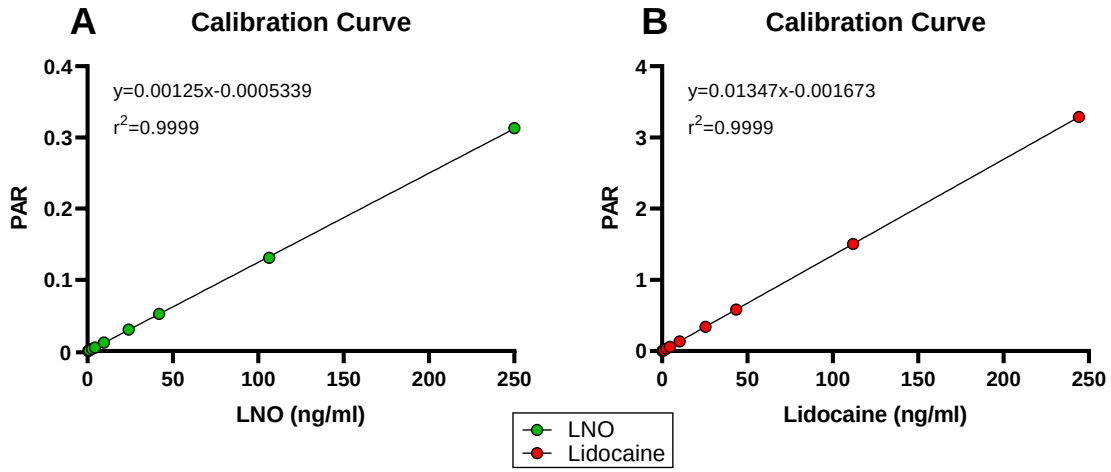


Figure 5.2 UHPLC-MS/MS calibration graphs – first protocol. A) LNO and B) lidocaine calibration line gradients and coefficient of determinations (r^2) calculated using linear regression analysis. PAR = peak area ratio. n=1 per point.

In LNO-perfused hearts, considerably less LNO was detected in ischaemic LV tissue compared with time-matched normoxic controls (Figure 5.3). However, the amount of lidocaine detected in ischaemic LV tissue was similar to that in time-matched normoxic controls (Figure 5.3). The lack of increase in lidocaine as a result of ischaemia in these hearts, relative to that in normoxic controls, was likely due to methodological problems that resulted in the inappropriate reduction of LNO in normoxic tissue during sample collection and/or preparation that meant that similar amounts of lidocaine were detected under the two conditions. Furthermore, the disappearance of LNO from ischaemic tissue, relative to normoxic controls, was not matched 1:1 with the appearance of lidocaine (Figure 5.3). The lack of 1:1 stoichiometry prompted concerns that the analysis method had resulted in an underestimation of lidocaine present in the tissue.

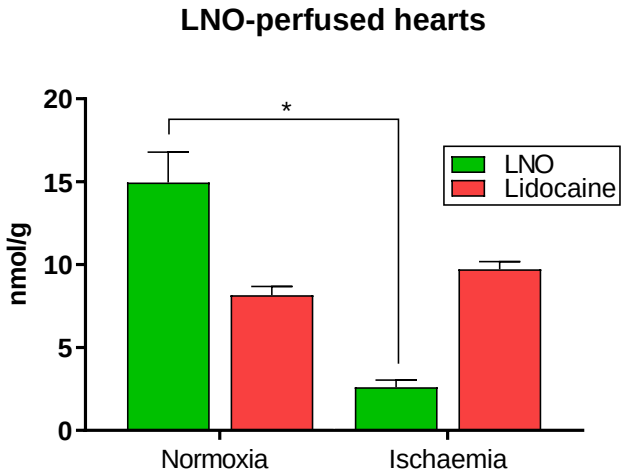


Figure 5.3 The presence of LNO and lidocaine in myocardial tissue – first protocol. LNO and lidocaine were detected using UHPLC-MS/MS methodology in myocardial tissue samples collected from Rat Langendorff-perfused isolated hearts perfused with 15 µM LNO after 30 min regional ischaemia or time-matched normoxia. n=6 per group; variables (mean ± SEM) were subjected to 1 way ANOVA followed by Tukey's post hoc tests (as F was significant and data Gaussian); *p<0.05 versus normoxic control.

5.4 Second protocol

5.4.1 Methods

In order to test whether some component of the sample analysis process had caused an underestimation of the lidocaine concentration, a further group of hearts was perfused with 15 µM lidocaine. To address a separate issue regarding the artefactual conversion of LNO to lidocaine in normoxic tissue, regional ischaemia followed by IZ dissection was replaced by global ischaemia and rapid whole heart freezing (a technique used to immediately halt metabolic reactions in myocardial tissue (Allison, Ramey and Holsinger Jr, 1978)) to prevent artefactual LNO reduction during sample *extraction*, and 2% H_2O_2, an oxidising agent, was added to the preparation mix in the round bottom 2 ml Eppendorf tubes to prevent artefactual LNO reduction during sample *preparation*.

Wistar rat (315-395 g) hearts were set up for Langendorff perfusion (section 2.1) with Krebs for 10 min. Perfusate was then switched to either 15 µM lidocaine (n=10) or 15 µM LNO (n=10) for 10 min. Hearts were then randomised to undergo either 30 min of global ischaemia by cessation of Krebs inflow (n=5/drug) (section 2.1.4) or 30 min time-matched normoxic perfusion (n=5/drug), during which all hearts were submerged in warmed Krebs (37°C) to maintain adequate tissue temperature and integrity (i.e. normothermic global ischaemia). After the 30 min protocol had elapsed, the surface of each heart was rinsed with warmed Krebs before being rapidly snap frozen with cryogenic tongs (cooled in liquid nitrogen to approximately -195°C). Samples of frozen ventricular tissue, weighing approximately 30 mg, were then dissected using a sterile scalpel and stored in CryoTube Vials (Thermo Scientific, US) in liquid nitrogen until subsequently prepared, along with 13 calibration standard samples, for UHPLC-MS/MS experimentation as in section 2.3 (Figure 5.4).

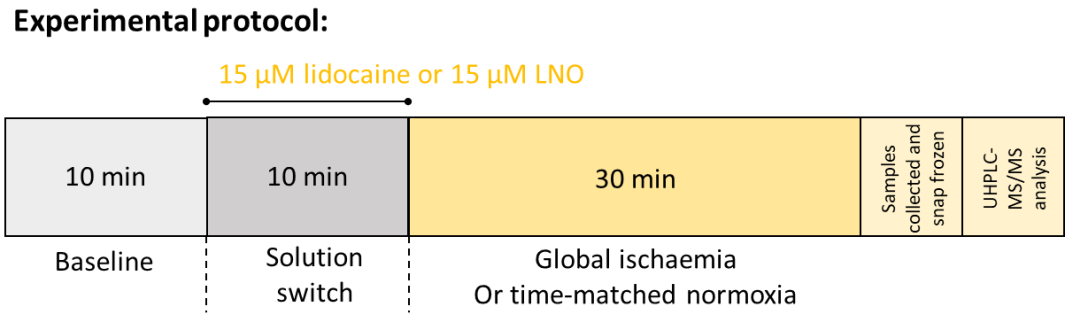


Figure 5.4 Diagram of the second experimental protocol followed to detect the presence of LNO and lidocaine in normoxic and ischaemic myocardial tissue from rat Langendorff-perfused isolated hearts.

5.4.2 Results

Standard calibration curves were constructed for lidocaine and for LNO on the same day that test samples were analysed. Detection was linear over the range 0-500 ng/ml and r^2 was 0.9992 for LNO (Figure 5.5A) and 0.9991 for lidocaine (Figure 5.5B). The correlation equations were y = 0.09135x + 0.005781 and y = 0.7155x + 0.004474 for LNO (Figure 5.5A) and lidocaine (Figure 5.5B), respectively.

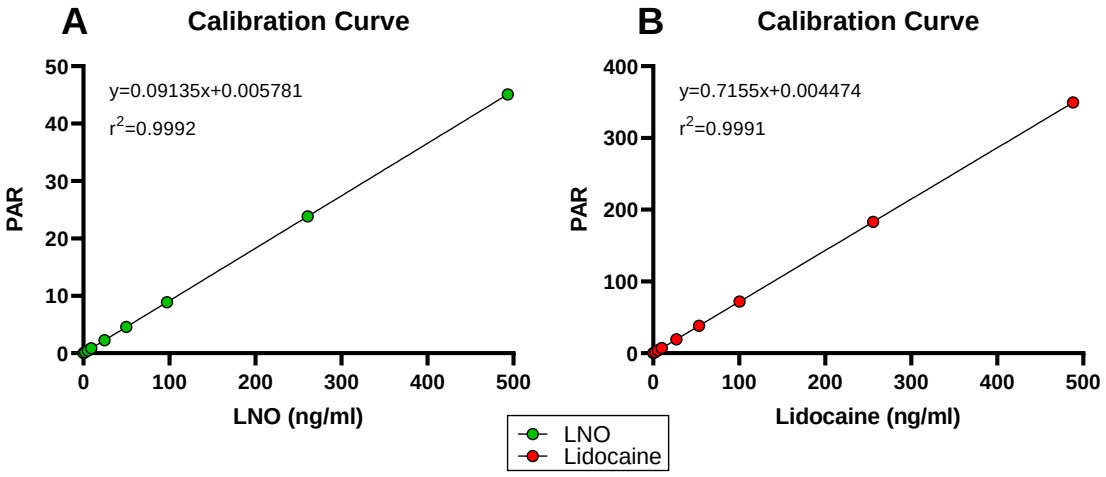


Figure 5.5 UHPLC-MS/MS calibration graphs – second protocol. A) LNO and B) lidocaine calibration line gradients and coefficient of determinations (r^2) calculated using linear regression analysis. PAR = peak area ratio. n=1 per point.

With the modified tissue processing, lidocaine became virtually undetectable in normoxic hearts perfused with LNO (<6% of the amount detected in ischaemic hearts) (Figure 5.6A). Global ischaemia caused an almost complete disappearance of LNO and the concomitant appearance of lidocaine (Figure 5.6A). This indicates that modified processing succeeded in avoiding artefactual conversion of LNO to lidocaine in normoxic tissue. However, the apparent stoichiometry of the conversion of LNO to lidocaine during ischaemia remained below 1:1 (Figure 5.6A) with only approximately 1/6th of the LNO replaced by lidocaine, equivalent to approximately 1/8th of the amount of lidocaine found in lidocaine-perfused hearts during ischaemia (Figure 5.6B). Additionally, a small amount of LNO was unexpectedly detected in lidocaine-perfused hearts during normoxia and ischaemia (Figure 5.6B), suggesting a small amount of lidocaine was oxidised to LNO during the sample collection or preparation steps, perhaps a consequence of the high concentration of H_2O_2 used in tissue processing.

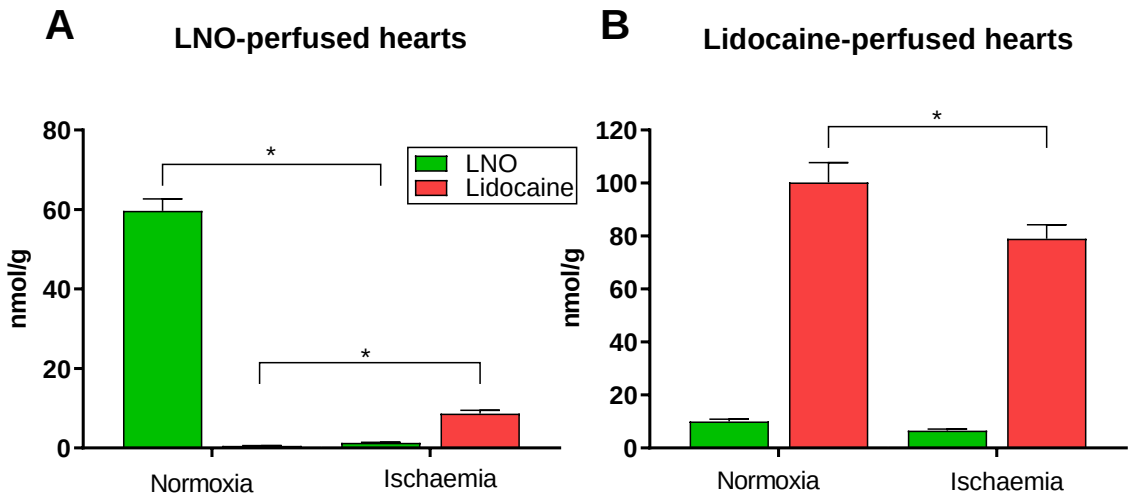


Figure 5.6 The presence of LNO and lidocaine in myocardial tissue – second protocol. LNO and lidocaine were detected using UHPLC-MS/MS methodology in myocardial tissue samples collected from rat Langendorff-perfused isolated hearts perfused with A) 15 µM LNO or B) 15 µM lidocaine after 30 min regional ischaemia or time-matched normoxia. n=6 per group; variables (mean ± SEM) were subjected to 1 way ANOVA followed by Tukey's post hoc tests (as F was significant and data Gaussian); *p<0.05 versus normoxic control.

5.5 Third protocol

5.5.1 Methods

The modifications in the second protocol had been of value, but, because small amounts of LNO had been detected in lidocaine-perfused hearts, a small-scale study was conducted to optimise H_2O_2 concentration; blank tissue samples were spiked with 100 ng lidocaine or 100 ng LNO, and H_2O_2 at 0%, 0.5%, 1% or 2%. The goal was to minimise the oxidation of lidocaine to LNO in lidocaine-spiked tissue whilst minimising the reduction of LNO to lidocaine in LNO-spiked tissue. This was achieved with 1% H_2O_2 (Figure 5.7).

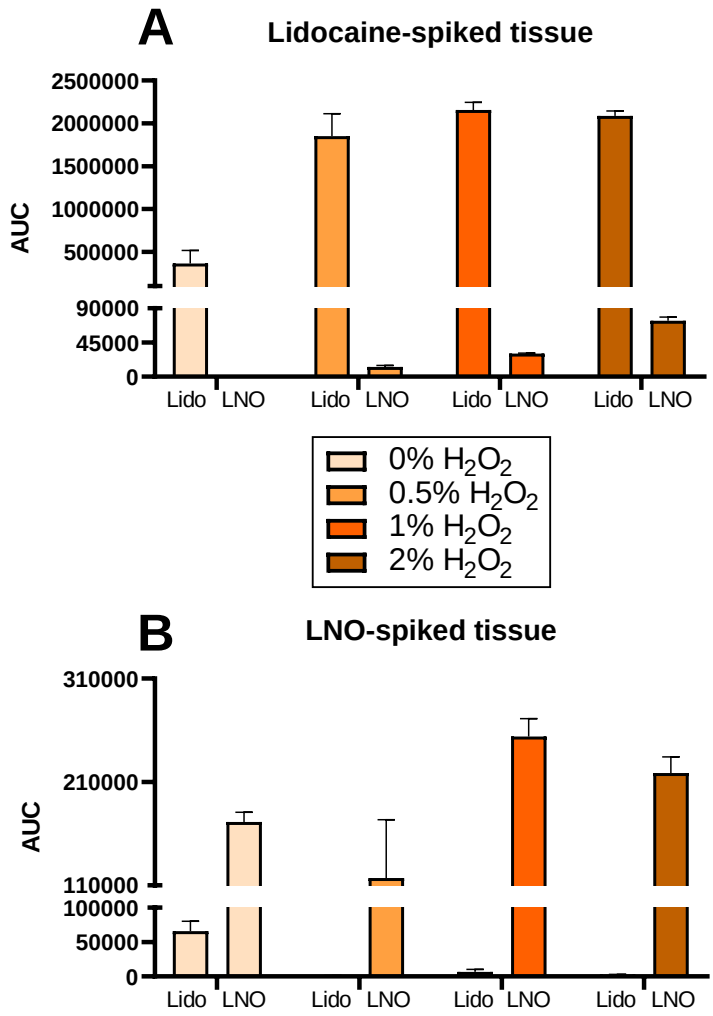


Figure 5.7 The effect of adding various H_2O_2 concentrations during sample preparation for UHPLC-MS/MS analysis. The presence of LNO and lidocaine, as detected using UHPLC-MS/MS methodology, in A) lidocaine-spiked myocardial tissue samples and B) LNO-spiked myocardial tissue samples when prepared using varying concentrations of H_2O_2 (0-2%) during tissue preparation and drug extraction. Lido = Lidocaine, AUC = Area under the curve. n=3 per group; mean ± SEM.

In addition, there is a possibility that the lack of 1:1 stoichiometry of LNO's conversion to lidocaine may be due to conversion of LNO to other products. If this is the case, the most obvious products to consider first are substances that are metabolites of lidocaine itself. Therefore, the UHPLC-MS/MS analysis MRM parameters were updated to permit the detection of MEGX, a major metabolite of lidocaine (Burney et al., 1974) which may also

contribute to lidocaine's therapeutic effect (Burney et al., 1974; Smith, Duce and Boyes, 1972) (Table 2.2). Additionally, in this study the duration of ischaemia was reduced from 30 min to 10 min to test the, albeit unlikely, possibility that peak conversion of LNO to lidocaine occurs earlier during ischaemia, after which lidocaine may be leached out of the IZ and, in regionally ischaemic hearts in which LNO's antiarrhythmic effect has been established (section 3.4.1), replaced by LNO (subsequently reduced to lidocaine) accessing the IZ via collateral vessels that are present in low amounts in rat hearts (Maxwell, Hearse and Yellon, 1987).

Wistar rat (315-394 g) hearts were set up for Langendorff perfusion (section 2.1) with Krebs for 10 min. Perfusate was then switched to either 15 µM lidocaine (n=10) or 15 µM LNO (n=10) for 10 min. Hearts were then randomised to undergo either 10 min of normothermic global ischaemia (n=5/drug) (section 2.1.4) or 10 min time-matched normoxic perfusion (n=5/drug), after which the surface of each heart was rinsed with warmed Krebs before being rapidly snap frozen with cryogenic tongs (all as described above). Samples of frozen ventricular tissue, weighing approximately 30 mg, were then dissected using a sterile scalpel and stored in CryoTube Vials (Thermo Scientific, US) in liquid nitrogen until subsequently prepared, along with 13 calibration standard samples, for UHPLC-MS/MS experimentation as in section 2.3 (Figure 5.8).

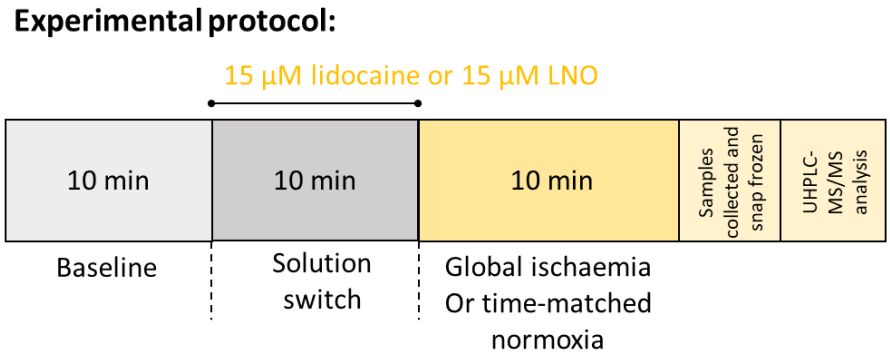


Figure 5.8 Diagram of the third experimental protocol followed to detect the presence of LNO, lidocaine and MEGX in normoxic and ischaemic myocardial tissue from rat Langendorff-perfused isolated hearts.

5.5.2 Results

Standard calibration curves were constructed for LNO, for lidocaine and for MEGX on the same day that test samples were analysed. Detection was linear over the range 0-500 ng/ml and the r^2 was 0.9996 for LNO (Figure 5.9A), 0.9996 for lidocaine (Figure 5.9B), and 0.9996 for MEGX (Figure 5.9C). The correlation equations were y = 0.0244x – 0.0277, y = 2.384x – 1.002 and y = 0.7563x – 1.094 for LNO (Figure 5.9A), lidocaine (Figure 5.9B) and MEGX (Figure 5.9C), respectively.

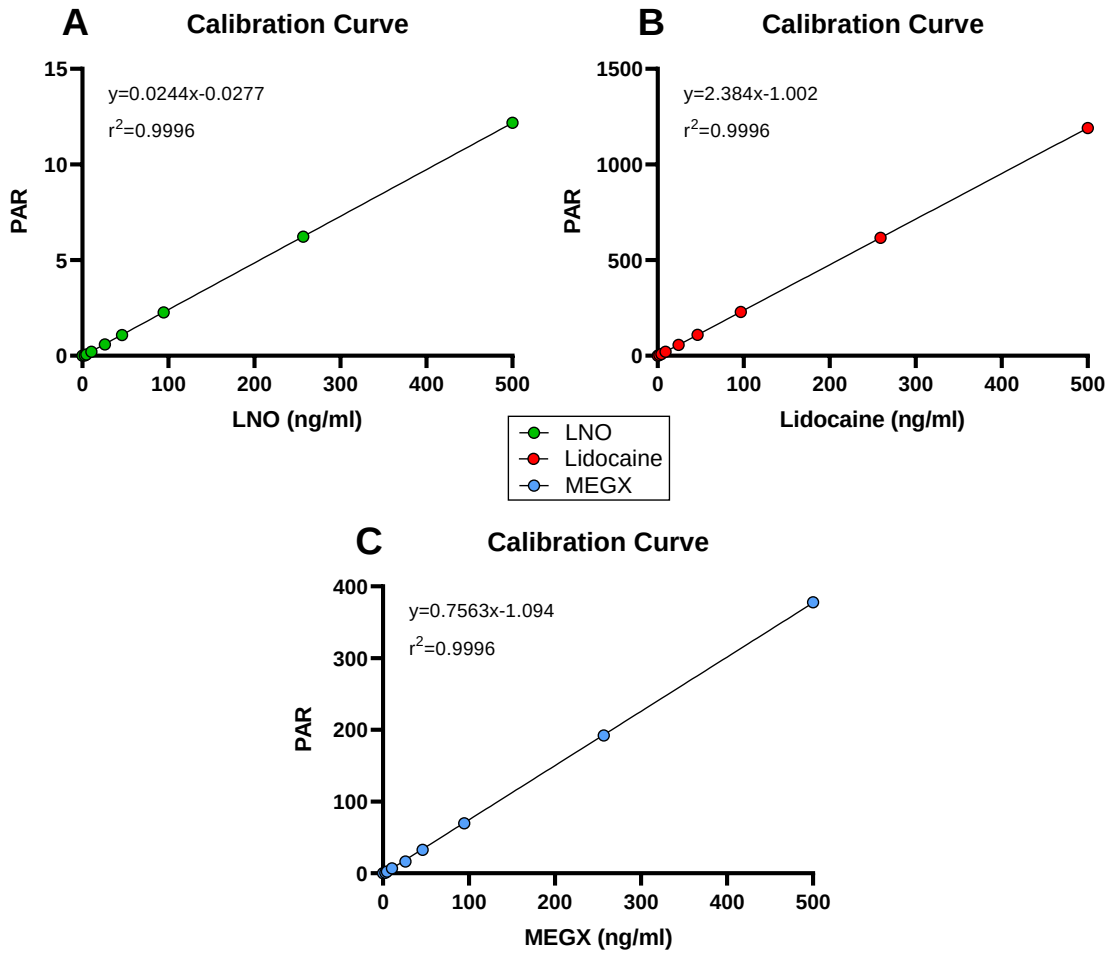


Figure 5.9 UHPLC-MS/MS calibration graphs – third protocol. A) LNO, B) lidocaine and C) MEGX calibration line gradients and coefficient of determinations (r^2) calculated using linear regression analysis. PAR = peak area ratio. n=1 per point.

Findings were similar to those in the second experimental protocol (section 5.4.2, Figure 5.6A+B), with an almost complete depletion of LNO from LNO-perfused hearts during ischaemia (Figure 5.10A) accompanied by the appearance of lidocaine (Figure 5.10A). The amount of lidocaine generated (Figure 5.10A) was similar to that seen previously (section 5.4.2, Figure 5.6A, Figure 5.6B), indicating that the duration of ischaemia (10 min versus the previous 30 min) was not a determining factor for the detection of lidocaine in LNO-perfused hearts. MEGX was detected, but only in small amounts in all tissue samples whether ischaemic or non-ischaemic and whether from LNO-perfused hearts or lidocaine-perfused hearts (Figure 5.11).

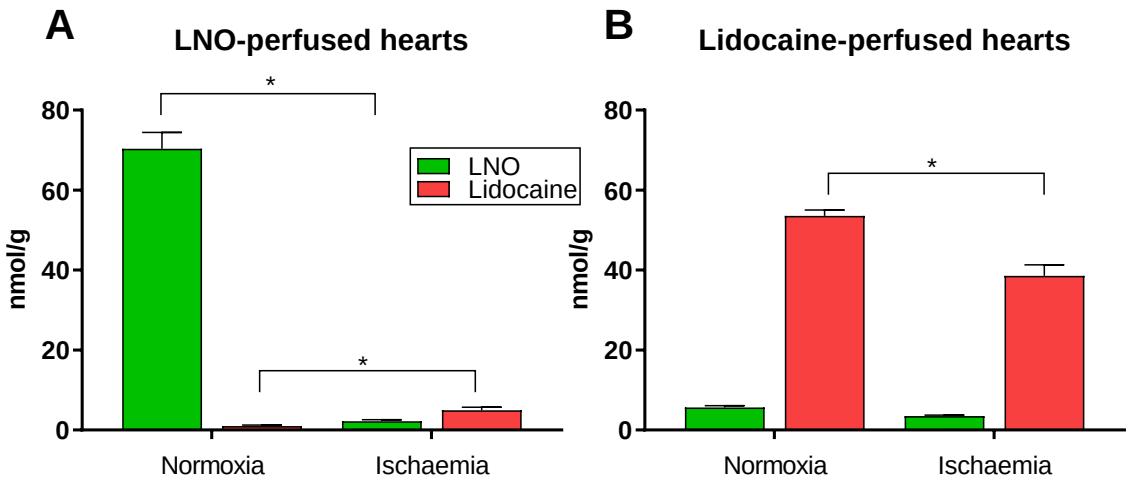


Figure 5.10 The presence of LNO and lidocaine in myocardial tissue – third protocol. LNO and lidocaine were detected using UHPLC-MS/MS methodology in myocardial tissue samples collected from rat Langendorff-perfused isolated hearts perfused with A) 15 µM LNO or B) 15 µM lidocaine after 10 min regional ischaemia or time-matched normoxia. n=6 per group; variables (mean ± SEM) were subjected to 1 way ANOVA followed by Tukey's post hoc tests (as F was significant and data Gaussian); *p<0.05 versus normoxic control.

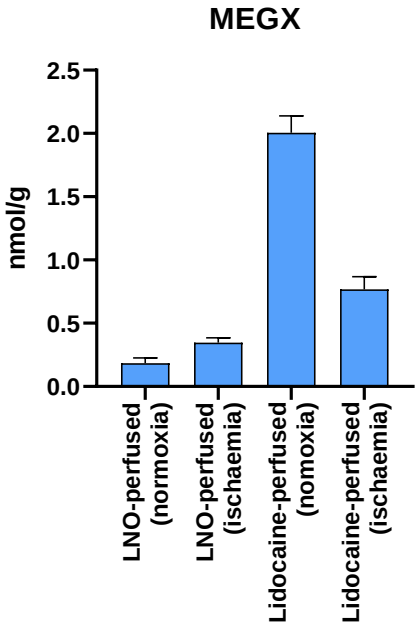


Figure 5.11 The presence of MEGX in myocardial tissue – third protocol. MEGX was detected using UHPLC-MS/MS methodology in myocardial tissue samples collected from rat Langendorff-perfused isolated hearts perfused with A) 15 µM LNO or B) 15 µM lidocaine after 10 min regional ischaemia or time-matched normoxia. n=6 per group; variables (mean ± SEM) were subjected to 1 way ANOVA followed by Tukey's post hoc tests (as F was significant and data Gaussian); *p<0.05 versus normoxic control.

5.6 Discussion

The objective of the present chapter was to develop an appropriate experimental protocol and UHPLC-MS/MS analysis method to examine and quantify the anticipated ischaemia-activated reduction of LNO to lidocaine in the rat myocardium. It was hypothesised that LNO would be sufficiently stable in the heart that it would not be reduced to lidocaine unless exposed to a period of myocardial ischaemia. In line with this, LNO was anticipated to be detected in high amounts in LNO-perfused normoxic tissue, with lidocaine appearing in place of LNO only once the tissue had become ischaemic.

There were key aspects of LNO's ischaemia-activated reduction which could be ascertained from the present results. LNO accumulated and was sufficiently stable (i.e. didn't reduce to

lidocaine) in the myocardium during normoxia. Ischaemia was associated with a loss of LNO and the appearance of lidocaine. Therefore, LNO was, as hypothesised, reduced to lidocaine only in the ischaemic myocardium. The similarity between the results obtained when the duration of ischaemia was set to either 10 or 30 min suggests that the reduction of LNO to lidocaine was complete by the 10^{th} min of ischaemia. Accordingly, any ischaemia-selective pharmacological action indicative of I_{Na} block presented in this thesis occurring with administration of LNO can be attributed to its ischaemia-activated conversion to lidocaine, which in some studies was evident as early as 5 min after ischaemia onset (section 4.4.2). Furthermore, these results align with unpublished results obtained *in vitro* in rat heart homogenate 22 years ago which showed substantial LNO depletion within 5 min of hypoxia, matched increasingly during 40 min of hypoxia by a rise in lidocaine (Tien, 1999).

The protocol employed for collecting the ischaemic and non-ischaemic heart tissue samples was adapted to achieve an accurate representation of each analyte present in the tissue at the time of sample extraction. The main changes made throughout optimisation were the addition of H_2O_2 into the preparation mix to ensure an oxidising environment and the utilisation of cryogenic tongs for rapid whole heart freezing. As a result, the detection of lidocaine during normoxia when hearts with perfused with LNO was almost entirely eliminated, suggesting that the initial detection of lidocaine under that condition had been an artefact of the initial sample extraction and preparation methods. In addition, a comparative group of lidocaine-perfused hearts was included to investigate whether the detection of lidocaine had been underestimated by the methodology, and this was determined to not be the case. Farkas and Curtis (2002) found that, in the rat Langendorff-perfused regionally ischaemic isolated heart, 3.88 µM lidocaine was ineffective at reducing ischaemia-induced VF. In the present study, relative to that detected in hearts perfused with 15 µM lidocaine, 15 µM LNO produced the equivalent of ~2 µM lidocaine in the ischaemic myocardium, insufficient, perhaps, to fully account for LNO's effect on ischaemia-induced VF (Farkas and Curtis, 2002). Although MEGX could theoretically contribute to a portion of LNO's antiarrhythmic effects if present in a high enough concentration, MEGX was detected in inconsequential amounts. The previous study presented in a PhD thesis (Tien, 1999), conducted *in vitro* in LNO-spiked hypoxic heart homogenate, rather than *ex vivo* in the rat

isolated heart, reported the yield of lidocaine from LNO to be approximately 50%. The low lidocaine yield in that study was also not accounted for by the presence of MEGX or 2,6-xylidine (another lidocaine metabolite) and no further explanation for the discrepancy was offered. In the present study, the low lidocaine yield was assumed to be the result of an unidentified technical limitation, since the tissue sampling and processing procedures and the ability of the methodology to detect lidocaine when added to the perfusion solution were all satisfactory. Further investigations to resolve this were considered beyond the scope and time frame of this PhD. With sufficient time one option would be to utilise a method called non-targeted metabolomics (Sévin et al., 2015). This technique is utilised to identify known and unknown metabolites and may be used in this context to investigate whether an unexplored lidocaine metabolite, or an undetected charged form of lidocaine, is instead present.

The key and important finding of the work presented in this chapter is that LNO was reduced to lidocaine in the ischaemic myocardium, a process which accounts for LNO's ischaemia-selective I_{Na} blocking actions. This accounts for the range of evidence that a compound with no pharmacological activity in normoxic tissue was able to produce a range of effects on arrhythmias, conduction and excitability in the presence of ischaemia. The mechanism of LNO's ischaemia-activated reduction shares similarities with other HAP compounds (Phillips, 2016; Mistry et al., 2017, section 1.4.1.2, Figure 1.5), beginning with a one electron reduction of a haemoprotein, catalysed by an NADPH-dependent P450 reductase, with which LNO becomes incorporated. After which the N-O bond is cleaved resulting in the release of an oxy-ferryl haemoprotein and lidocaine. Finally, a further one electron reduction of the oxy-ferryl haemoprotein and the addition of $2H^+$ results in the formation of a haem[Fe^{III}] and water (Tien, 1999). This reduction is inhibited by the presence of oxygen (Mistry et al., 2017; Wilson and Hay, 2011; Tien, 1999), hence the ischaemia-selectivity of LNO's actions.

The antiarrhythmic drug, verapamil, can inhibit ischaemia-induced VF in rat hearts when administration begins after coronary ligation, and effective extraction procedures allow the accumulation of verapamil in the IZ to be quantified (Curtis, MacLeod and Walker, 1984). LNO inhibited ischaemia-induced VF even when administration began after the start of regional

ischaemia (chapter 3), but owing to the need to use global ischaemia and freezing clamping when measuring LNO's conversion to lidocaine it was not possible to evaluate LNO accumulation (via collateral vessels) and conversion to lidocaine in the IZ when administration was begun after the start of ischaemia.

5.7 Summary

LNO was identified in the normoxic myocardium. Ischaemia caused a loss of LNO and the appearance of lidocaine. Therefore, LNO was, as hypothesised, reduced to lidocaine in the myocardium exclusively during ischaemia.

Chapter 6: General discussion and conclusions

6.1 Summary of aims and methodology

Little attention has been paid in recent decades to the development of novel drugs to prevent ischaemia-induced ventricular arrhythmias, and yet no antiarrhythmic drug is currently considered safe and effective enough to permit widespread use in this context. Despite the preventative measures available to those identified as 'at risk' of SCD (e.g. statins, β-blockers, ICD implantation), the rate of ischaemia-induced VF and SCD in these populations remains high, and SCD itself contributes to 15-20% of deaths worldwide. Therefore, novel antiarrhythmic drugs are required to complement non-pharmacological interventions in high SCD risk patients (Sossalla, 2017), and provide larger lower risk populations with a safe option for arrhythmia prevention, with the ultimate aspiration to operate as a primary and secondary prophylactic drug treatment in diverse risk populations (Wellens et al., 2014; Myerburg et al., 1998; Huikuri, Castellanos and Myerburg, 2002). The overarching aim of this PhD thesis was to examine a putative novel ischaemia-activated antiarrhythmic prodrug called LNO. Prior to this PhD, LNO had been synthesised by the N-oxidation of lidocaine (Patterson et al., 1986; Craig and Purushothaman, 1970) and the mechanism underpinning its hypoxia-activated reduction to lidocaine had been characterised through *in vitro* experimentation (Tien, 1999), but its antiarrhythmic capabilities and mechanism of action had not been examined.

Established *ex vivo* and *in vivo* rat models of myocardial ischaemia were utilised in a range of experimental protocols to examine LNO's pharmacological actions. Firstly, an examination of LNO's TTI relative to that of existing class 1b antiarrhythmic drugs was required. This was assessed using regional ischaemia in the rat Langendorff-perfused isolated heart and *in vivo* in the anaesthetised rat, both established models for studying the effects of drugs on ischaemia-induced arrhythmias (Curtis, 1998). Antiarrhythmic actions and cardiovascular ADRs (e.g. bradycardia, PR interval prolongation, negative inotropy) were studied in tandem in the rat isolated heart. Subsequently, estimates of LNO's TTI were refined using the anaesthetised rat to recapitulate its antiarrhythmic effect and (using cumulative drug boluses)

to identify LNO's threshold dose for acute *in vivo* adversity (e.g. effects on cardiac output and ECG variables) compared with lidocaine.

Subsequently, evidence was sought to verify the accumulating evidence that LNO was reduced to lidocaine selectively during ischaemia. The rat Langendorff-perfused regionally ischaemic isolated heart model was used to investigate LNO's electrophysiological actions during ischaemia and normoxia in comparison to lidocaine, seeking to identify whether outcomes indicative of ischaemia-selective I_{Na} block were responsible for LNO's antiarrhythmic capabilities. This utilised epicardial pacing and optical mapping techniques to measure drug-induced changes to the ERP, conduction velocity and the occurrence of rate-dependent conduction block in the rat heart. In addition, a rat isolated heart perfusion protocol and tissue sampling and processing method was developed and optimised to enable the identification of lidocaine and LNO in the normoxic and ischaemic myocardium, utilising a newly established UHPLC-MS/MS analysis method.

Each study was discussed in the relevant sections of each thesis chapter. Herein, a summary and conclusion of the main findings is made and limitations and avenues for future experimental work and drug development are discussed.

6.2 Summary of findings

6.2.1 Studies characterising the TTI of LNO

In rat Langendorff-perfused isolated hearts with regional ischaemia, LNO was as effective as lidocaine at preventing ischaemia-induced VF. This was the case irrespective of whether drug administration began before or after ischaemia onset, although a higher concentration of LNO was required to elicit an antiarrhythmic effect when delivered post-ligation only. In this model, antiarrhythmic concentrations of lidocaine caused archetypal class 1b antiarrhythmic drug-induced ADRs (e.g. bradycardia, PR interval prolongation and negative inotropy) mediated in non-ischaemic and supraventricular regions of the myocardium, whereas LNO was without effect up to 4 fold its antiarrhythmic concentration, demonstrating a preliminary

TTI of >4. In order to refine LNO's TTI further, the antiarrhythmic effectiveness of LNO and lidocaine was recapitulated *in vivo* in the anaesthetised rat with regional ischaemia, in which i.v. administration of lidocaine or LNO prevented ischaemia-induced VF and improved the survival rate to the same extent. And, in the *in vivo* cumulative dose toxicity study, lidocaine caused cardiovascular ADRs (e.g. bradycardia, PR interval prolongation and cardiac output reduction) at a threshold dose lower than that required to elicit its antiarrhythmic effect. In contrast, LNO was devoid of such actions up to 30 times its antiarrhythmic dose. Therefore, whilst lidocaine displayed no discernible TTI (a similar case to that observed with mexiletine in published studies), LNO's TTI was found to be >30.

6.2.2 Studies elucidating the ischaemia-selective antiarrhythmic mechanism of LNO

In rat Langendorff-perfused isolated hearts paced at a sinus rate and loaded with di-4-ANEPPs, LNO and lidocaine had no adverse effect on myocardial conduction velocity (as measured by optical mapping) in the absence or presence of regional ischaemia. In addition, in a separate experiment in rat Langendorff-perfused isolated hearts paced at a sinus rate, the ERP was consistently prolonged by lidocaine during regional ischaemia. LNO, however, did not prolong the ERP. Therefore, taken together, the slowing of myocardial conduction velocity or prolongation of ERP at a sinus rate does not play a role in the antiarrhythmic mechanism of LNO.

In a subsequent study in the rat Langendorff-perfused isolated heart, the rate-dependence of the drugs' effects on myocardial conduction was examined. Lidocaine caused ischaemia-independent conduction slowing and rate-dependent conduction block when hearts were paced at a rate >600 beats/min, whereas LNO had no effect on conduction in the absence of ischaemia. After ischaemia onset, myocardial conduction slowed substantially in controls within 5 min, and was further slowed as ischaemia progressed. At 5 and 10 min into ischaemia, rate-dependent conduction block occurred in control hearts predominately at high pacing rates (>700 beats/min). LNO and lidocaine potentiated ischaemia-induced conduction slowing and hastened the time for conduction block to occur, such that it occurred

predominantly at lower pacing rates (400-600 beats/min), at 5 and 10 min into ischaemia. After 20 min of ischaemia, rate-dependent conduction block occurred in a similar pattern in the drug groups and controls. The actions displayed by lidocaine and LNO were expected as a result of cumulative beat-to-beat I_{Na} block, with the distinction between LNO and lidocaine being the absolute dependency on the presence of ischaemia for LNO's actions. These observations demonstrate that LNO is pharmacologically inactive in the absence of ischaemia and is reduced to a pharmacologically active I_{Na} blocking antiarrhythmic agent exclusively during ischaemia.

6.2.3 Studies providing evidence of the ischaemia-activated conversion of LNO into lidocaine

Samples of myocardial tissue extracted from rat Langendorff-perfused isolated hearts after a period of either 10 or 30 min global ischaemia, or time-matched normoxia, were analysed using a newly developed UHPLC-MS/MS method. The introduction of measures such as freeze clamping and H_2O_2 during tissue sampling and processing were successful in preventing artefactual reduction of LNO in normoxic tissue. Global ischaemia was associated with a complete depletion of LNO, levels of which were high in normoxic tissue, and an increase in detectable of lidocaine, which was virtually undetectable in normoxic tissue. The stoichiometry of LNO's reduction to lidocaine was not 1:1 and, despite efforts, this discrepancy was not resolved. Nevertheless, the key finding herein was that LNO was indeed converted to lidocaine selectively in ischaemic tissue, supporting the central hypothesis of this thesis.

6.3 General conclusions

From the results presented in this thesis, it can be concluded that LNO is pharmacologically inactive and stable in the normoxic rat heart, becoming pharmacologically active exclusively during myocardial ischaemia. During ischaemia, LNO is reduced to lidocaine and this is sufficient to account for its pharmacological actions on VF and other variables, actions all of which mimic those of lidocaine and are attributable to ischaemia-selective I_{Na} block. LNO

caused no ADRs, even at a dose 30 times its antiarrhythmic dose, resulting in TTI >30. In contrast, lidocaine had a TTI no greater than 1, because it caused ADRs at a threshold dose lower than its antiarrhythmic dose. These results justify further preclinical and clinical development of LNO as an ischaemia-activated antiarrhythmic prodrug. The N-oxide modification to lidocaine in order to synthesise LNO evidently affords the drug with the necessary ischaemia-selectivity required to overcome a central issue concerning the use of lidocaine and other antiarrhythmic drugs developed for the purpose of preventing ischaemia-induced arrhythmogenesis; their adversity (Walker and Guppy, 2003).

6.4 Future work

6.4.1 Direct measurement of ischaemia-selective I_{Na} block

LNO's antiarrhythmic mechanism was attributed to ischaemia-selective I_{Na} block. However, this inference was based upon measurements obtained at the whole heart level (i.e. hastened rate-dependent conduction block) and the knowledge that I_{Na} block during ischaemia is the antiarrhythmic mechanism of lidocaine (section 1.3.2.5). Therefore, obtaining direct evidence of LNO's effect on I_{Na} conductance and its Na^+ channel binding at the molecular level would be an important future endeavour. As described in section 1.3.2.5.2, lidocaine binds to the $Na_v1.5$ channel in its *I* state at a high affinity binding site within the channel's pore, interacting with two residues, Phe1760 and Tyr1767, in the channel's DIVS6 segment (Fozzard, Sheets and Hanck, 2011; Pless et al., 2011; Nguyen et al., 2019). This binding leads to the inhibition of I_{Na} and determines lidocaine's use-dependent and voltage-dependent blocking properties (Lee et al., 1981). In addition, lidocaine interferes with the channel's gating mechanism, slowing recovery from the channel's *I* state (Fozzard, Sheets and Hanck, 2011). Indeed, if LNO's ischaemia-dependent pharmacological effects are the result of an ischaemia-activated bioreduction to lidocaine and resultant I_{Na} block, then investigations involving isolated ventricular myocytes and patch clamp recordings of $Na_v1.5$ (Lee et al., 1981; Kornreich, 2007) under normoxic conditions would reveal no inhibition of I_{Na} upon application of LNO. In contrast, if the same preparation was set up in a hypoxic environment, then application of

LNO would result in inhibition of I_{Na} to a similar extent to that achieved by application of lidocaine itself. Hypoxia in isolated cellular preparations typically involves either perfusion with a buffer mimicking the conditions of ischaemia (e.g. hyperkalaemia, acidosis, gassed with 90/95% N_2 and 10/5% CO_2 etc.) (Louch, Ferrier and Howlett, 2002), by utilising atmospheric chambers (Safran et al., 2001), or a combination of both. LNO would also be expected to interact with the Na^+ channel's high affinity binding site exclusively during ischaemia in a manner identical to that of lidocaine (section 1.3.2.5.2). Therefore, experiments utilising site-directed mutagenesis at the Phe1760 and Tyr1767 residues (Pless et al., 2011), combined with patch clamp recording (e.g. Friederich et al., 2004), could be conducted to investigate this.

However, there are potential complications to consider that may arise when conducting patch clamp recordings whilst attempting to maintain a hypoxic environment. Simultaneous patch clamp recording and use of a closed atmospheric chamber may prove technically challenging, and perfusing myocytes with an 'ischaemic' solution in an open system is inherently vulnerable to ambient O_2 and may not accurately replicate all aspects of ischaemia present in the whole heart and *in vivo* (Pitts and Toombs, 2004) or the optimal conditions for LNO's reduction. Despite these challenges, performing experiments to confirm that LNO blocks I_{Na} during ischaemia, and shares a common binding site with lidocaine, would be a valuable next step.

6.4.2 Extension of findings to further species

The rat heart was the obvious first choice study species because it has numerous advantages as a model of myocardial ischaemia, including the reproducibility of the IZ size (Ridley, Yacoub and Curtis, 1992; Wilder et al., 2016), the predictability of the resultant time-course of ischaemia-induced arrhythmogenesis (Curtis, Macleod and Walker, 1987; Curtis, 1998) and the facility to allow simultaneous monitoring of cardiac ADRs (Hesketh et al., 2020). However, there are limitations of the rat species that may justify planning work in a second species.

The high heart rate in rats may exacerbate the use-dependent effects of lidocaine on heart rate and the PR interval compared to other species (Kodama et al., 1987; Matsubara, Clarkson and Hondeghem, 1987). Other species with a lower basal sinus rate, such as the guinea pig,

cat or dog (Farmer and Levy, 1968), may therefore exhibit less pronounced lidocaine-induced cardiovascular ADRs, thus explaining the species discrepancies discussed in section 3.4.2. This is of most concern for class 1c and 1a drugs, whose I_{Na} blocking capabilities are characterised by slower binding and unbinding rates (Hondeghem and Katzung, 1984), but still warrants consideration with regards to class 1b drugs (Hondeghem and Katzung, 1984). However, given that LNO was without effect on any ECG variable studied presently in the rat heart, wherein effects may be exaggerated, it is likely the LNO would also be devoid of such actions in other animal models and in humans.

The rat heart also lacks certain repolarisation currents (I_k comprised of I_{kr} and I_{ks}) (Tande et al., 1990), that are functionally important in other species (Rees and Curtis, 1996). Repolarisation in the rat heart is instead mediated by the transient outward current (I_{to}) (Josephson, Sanchez-Chapula and Brown, 1984), inwardly rectifying current (I_{k1}) (Josephson and Brown, 1986) and K_{ATP} (Wolleben, Sanguinetti and Siegl, 1989). Therefore, drugs that selectively block I_k have no effect on repolarisation variables such as APD (Tande et al., 1990) or the occurrence of ischaemia-induced VF (Rees and Curtis, 1993) in the rat heart. Whilst this is not an issue when investigating the antiarrhythmic mechanism of class 1b drugs, I_{kr} blockade is a recognised mechanism of proarrhythmia (Hondeghem and Snyders, 1990). Lidocaine itself, however, has a very low inhibitory potency for the channel that mediates I_{kr} (Du et al., 2014; Paul, Witchel and Hancox, 2002), and therefore LNO, an ischaemia-selective lidocaine mimetic, is very unlikely to block I_{kr} to a degree that warrants concern. Confirmation of this, however, would be a valuable investigation to undertake in a species with more human-like repolarisation characteristics (Rees and Curtis, 1996).

Finally, non-cardiac adversity with LNO self-evidently can't be measured in the rat Langendorff-perfused isolated heart and was not the focus of acute *in vivo* investigations in the anaesthetised rat. Addressing concerns about possible non-cardiac ADRs is important when a new drug has been found to be devoid of cardiac ADRs in the isolated heart, and thus a large preliminary TTI has been established (Hesketh et al., 2020), as is the case with LNO. The potential for LNO to induce ADRs of non-cardiovascular origin would need to be subsequently investigated *in vivo* in multiple animal species.

Given the above, it is therefore important to recapitulate the present results and further interrogate cardiac and non-cardiac ADRs in a second species which possesses more human-like haemodynamic and electrophysiological properties compared with the rat. Selection of a second species is, however, difficult owing to the poor reproducibility and/or high cost of other approaches (Curtis, 1998). The rabbit has a lower basal sinus rate (Coker, 1989), a longer ventricular APD and plateau (Varró et al., 1993), and more human-like regulation of Ca^{2+} movement and excitation-contraction coupling (Bers, 2002). However, it is not often used, and its best characterisation reveals it to be variable in outcome (Coker, 1989; Bril, Forest and Gout, 1991), clearly inferior to the rat as a bioassay. Nevertheless, of a range of possible options, the rabbit may be the best 'second species' to the rat. Lidocaine's known profile of electrophysiological effects during ischaemia has indeed been reproduced in the 'working' rabbit isolated heart (Wolk et al., 1998). Whilst parallel monitoring of potential ADRs (e.g. bradycardia and PR interval prolongation) may be conducted during a second species investigation into antiarrhythmic effectiveness, replicating the *in vivo* anaesthetised rat cumulative dose toxicity study in the anaesthetised rabbit would provide a focused and accurate account of LNO's ECG and haemodynamic ADRs in a second species. Although unlikely, if LNO did produce I_{kr} block, this would manifest as APD (i.e. QT interval) prolongation in the rabbit (Lu et al., 2001), and would warrant further safety investigations into its mechanism and implication.

6.4.3 Further studies using rats

To further explore possible non-cardiac ADRs, conscious rats may be used for additional acute toxicity studies to compare the penetration of lidocaine and LNO into the CNS, and the occurrence of convulsions. This method has previously been used to demonstrate that mexiletine readily passes into, and accumulates in, the CNS wherein it causes observable convulsions at an effective antiarrhythmic dose (Igwemezie et al., 1992). Similarly, lidocaine has also been shown to induce convulsions in conscious rats, although the dose administered was high (Barrett, Hayes and Walker, 1995). Therefore, comparing the threshold dose for CNS adversity for LNO and lidocaine may also be used to further refine the TTI of LNO.

6.4.4 Translation to the clinical setting

Successful application of LNO in the clinical setting could see widespread use of the drug in a combination of individuals at a high risk of SCD, as an adjunct to non-pharmacological antiarrhythmic therapy, and larger cohorts of patients with lower SCD risk profiles. When developing novel antiarrhythmic drugs, the entry level patient group often comprises AMI survivors, a patient population at risk of death from non-arrhythmic causes and arrhythmic causes (Yap et al., 2005), the latter of which may be the result of heart failure or infarct related arrhythmias (i.e. phase 2) or arrhythmias arising due to recurrent ischaemic events (i.e. phase 1) (Jokhadar et al., 2004; Adabag et al., 2008). There is evidence that ischaemia and infarction evoke lethal arrhythmias by differing mechanisms (sections 1.2.2), with differing susceptibility to the action of drugs, manifesting as VF suppression during acute ischaemia and proarrhythmia during infarction or vice versa (Curtis, 1998; Clements-Jewery, Hearse and Curtis, 2005), rendering appropriate patient selection for initial clinical studies an important determinant of trial outcome. Given the data presented in this thesis, ischaemia is clearly necessary for LNO to exhibit its antiarrhythmic effect and the present dataset only addresses the effectiveness of LNO against phase 1 arrhythmias, not those occurring in phase 2. Therefore, for initial clinical efficacy assessment it is suggested that testing should involve acute i.v. administration of LNO by paramedics to patients with ST-elevation AMI (Ibanez et al., 2018; Dan et al., 2018). Indeed, data from this thesis shows that administration of LNO after ischaemia onset is a viable option (section 3.4.1.2). By focussing initial efforts on establishing effectiveness and safety in a homogeneous population that is guaranteed to have a high incidence of ischaemia-induced VF, a positive outcome in this context will provide the justification for the more costly expansion into testing effectiveness in heterogenous high risk populations (i.e. in patients post-MI or qualifying for an ICD) and in larger lower risk populations meeting reasoned criteria for the onset of ischaemia-induced VF (Huikuri, Castellanos and Myerburg, 2002; Myerburg et al., 1998), with the eventual aspiration to provide LNO as a primary intervention to anyone identified as ‘at risk’. The latter would rely on robust risk stratification measures to identify potential beneficiaries (Wellens et al., 2014; Priori et al., 2015).

6.5 Considerations for future drug development

Whilst the data outlined in this thesis are promising, the variable success of HAPs in cancer therapy has highlighted a few broad concepts to consider moving forward. Firstly, there is a delivery issue inherent to HAPs, since these drugs are activated in difficult to access target tissues with little surrounding functional blood vessels (Mistry et al., 2017). LNO has demonstrated it can be administered after ischaemia onset and penetrate target tissue, even in rat hearts where collateral blood supply is low, however, to permit chronic use outside the hospital setting, an oral or easily absorbed mucosal preparation must be developed. This clinical formulation must have pharmacokinetic properties that permit the drug to be sufficiently stable *in vivo* and able to reach the target tissue efficiently, whilst ensuring site-selective reduction is rapid and leads to a high lidocaine yield. Secondly, following the preliminary success of LNO, there is justification to consider additional antiarrhythmic drug candidates to undergo N-oxide modification. I_{CaL} blockers, despite their effectiveness against ischaemia-induced VF in animal models, are limited in humans by their indiscriminate actions in the vasculature and heart at therapeutic doses (section 1.3.2.4). The ideal I_{CaL} blocker for use against ischaemia-induced VF would be one that worked selectively during ischaemia and exhibited tachycardia-selectivity (Curtis, MacLeod and Walker, 1984), and thus a diltiazem N-oxide or verapamil N-oxide ischaemia-activated prodrug may represent a focus for subsequent antiarrhythmic drug development. In this, it is important to consider the stability of any newly developed N-oxide *in vivo* and whether the CYP450 enzymes involved in its reduction pathway are expressed in the human ventricle, as was studied extensively in LNO's conception (Tien, 1999). And, lastly, novel HAPs may be of interest to other fields of drug development, specifically those in which hypoxia is characteristic to the disease pathology (i.e. stroke).

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