Ipratropium Bromide-Mediated Myocardial Injury in In Vitro Models of Myocardial Ischaemia/Reperfusion

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Abstract

Ipratropium bromide (a non-selective muscarinic antagonist) is widely prescribed for the treatment of Chronic Obstructive Pulmonary Disease (COPD). Analyses of COPD patients, with underlying ischaemic heart disease (IHD), receiving anticholinergics, have indicated increased risk of severity and occurrence of cardiovascular events (including myocardial infarction, (MI)). The present study explored whether ipratropium bromide induces myocardial injury in non-clinical models of simulated myocardial ischaemia/reperfusion injury. Adult Sprague Dawley rat hearts/primary ventricular myocytes were exposed to simulated ischaemia/hypoxia prior to administration of ipratropium at the onset of reperfusion/reoxygenation. Infarct to risk ratio and cell viability was measured via triphenyl tetrazolium chloride staining and thiazolyl blue tetrazolium bromide (MTT) assay. The involvement of apoptosis and necrosis were evaluated by flow cytometry. Mitochondrial-associated responses were detected by tetramethyl rhodamine methyl ester (TMRM) fluorescence and myocyte contracture. Ipratropium ($1x10^{-11}$ M - $1x10^{-4}$ M) significantly increased infarct/risk ratio and decreased cell viability, in a dose-dependent manner. Increased levels of necrosis and apoptosis were observed via flow cytometry, accompanied by increased levels of cleaved caspase-3 following ipratropium treatment. Levels of endogenous myocardial acetylcholine were verified via use of an acetylcholine assay. In these experimental models, exogenous acetylcholine ($1x10^{-7}$ M) showed protective properties, when administered alone, as well as abrogating the exacerbation of myocardial injury during ischaemia/reperfusion following ipratropium co-administration. In parallel experiments, under conditions of myocardial ischaemia/reperfusion, similar injury was observed following atropine ($1x10^{-7}$ M) administration. These data demonstrate for the first time in a non-clinical setting that ipratropium exacerbates ischaemia/reperfusion injury via apoptotic and necrotic associated pathways.

Key words

Introduction

Ipratropium bromide ([8-methyl-8-(1-methylethyl)- 8-azoniabicyclo[3.2.1] oct-3-yl] 3-hydroxy-2-phenyl-propanoate, ipratropium), a non-selective muscarinic receptor antagonist (Wood *et al.*, 1995), has been widely prescribed for management of Chronic Obstructive Pulmonary Disease (COPD) since 1987 (Restrepo, 2007) and more recently as adjunctive emergency treatment for acute asthma (Aaron, 2009). The antagonist action of ipratropium at the muscarinic receptors in the lungs (primarily M_3 subtype) inhibits acetylcholine induced reflexes, leading to smooth muscle relaxation. This facilitates airflow by alleviating the bronchospasm symptomatic of COPD exacerbations (Tranfa *et al.*, 1995; Restrepo, 2007).

There are five known subtypes of muscarinic receptors (mAChRs), distributed across a plethora of tissues. MAChRs are responsible for a multitude of neuronal and non-neuronal actions (Caulfield and Birdsall, 1998; Resende and Adhikari, 2009). Within mammalian pulmonary systems, the M_1, M_2, M_3 and M_4 subtypes are all differentially expressed (Wessler and Kirkpatrick, 2008). Although M_2 receptors constitute the majority of mAChRs in the myocardium, it has been shown that M_1 and M_3 receptors are also present. In particular, it has been shown that activation of M_3 receptors may be associated with activation of pro-survival signalling cascades following ischaemia/reperfusion (I/R) (Yue *et al*., 2006; Wang *et al*., 2007).

In addition to airflow obstruction, there is increasing evidence that COPD contributes to systemic pathologies, including Ischaemic Heart Disease (IHD) (Macnee *et al*., 2008). Underlying IHD also represents the highest risk factor for death by myocardial infarction (MI) in COPD sufferers (Zielinski *et al*., 1997). It is estimated that approximately 22% of patients with COPD suffer from underlying IHD.

Within the past decade, despite much controversy, numerous studies have indicated cardiovascular risks associated with COPD patients receiving anticholinergic therapies (Singh *et al.,* 2008). However, despite the known co-morbidities, to our knowledge there

have been no non-clinical investigations to date to assess the effects of anticholinergic compounds, such as ipratropium, in the setting of simulated IHD or MI. Further to this, Shaik *et al* (2012) have recently demonstrated that ipratropium is capable of eliciting suicidal erythrocyte death (eryptosis) via a mechanism due to stimulation of increased cytosolic Ca^{2+} activity (Shaik *et al.*, 2012). Despite differences between eryptosis and apoptosis, which only occurs in nucleated cells, both of these processes can be initiated by increases in Ca^{2+} concentration, indicating that ipratropium may have the ability to also trigger apoptosis.

Conversely, endogenous muscarinic activation by acetylcholine has revealed cytoprotective properties against various cellular insults, including conditions of myocardial I/R injury (Budd *et al.*, 2003; De Sarno *et al.*, 2003; Critz *et al.*, 2005; Resende and Adhikari, 2009; Li *et al.*, 2011).

The exact mechanism by which I/R injury occurs remains a matter of some debate, however it is known that myocyte loss occurs via both apoptosis and necrosis (Kung *et al.*, 2011). The key determinant for initiation of intrinsic apoptosis is mitochondrial outer membrane permeabilisation (MOMP), thereby initiating a caspase-dependent apoptotic cascade (Dewson and Kluck, 2009). Acetylcholine has been shown effective in protecting the myocardium against hypoxia induced apoptosis (Kim *et al.*, 2008; Liu *et al.*, 2011) by regulation of Bcl-2 family proteins and caspase-3, a pivotal caspase in the machinery leading to apoptotic death. In the context of a healthy cell, caspases exist as inert zymogens; however, during apoptosis proteolytic cleavage renders them active, permitting caspase-dependent apoptosis.

During I/R, increases of intracellular of Ca^{2+} and oxidative stress lead to rapid ATP depletion, thereby causing rupture of the outer mitochondrial membrane. This facilitates the influx of toxic mitochondrial proteins into the cytosol, thus initiating apoptosis (Hausenloy *et al.*, 2002; Halestrap and Pasdois, 2009) as well as myocyte death by necrosis (Yellon and Hausenloy,

2007) which occurs via the resultant collapse of mitochondrial function after disturbance of mitochondrial membrane potential (Crompton, 1999).

The purpose of this study was to determine the effect of ipratropium bromide administration in the context of I/R in isolated perfused rat heart (1×10^{-9} M – 1×10^{-6} M) and simulated I/R in a primary rat cardiac myocyte model (1×10^{-11} M – 1×10^{-4} M). Acetylcholine (1×10^{-7} M) was also administered concomitantly with ipratropium to ascertain whether the observed effects of ipratropium were due to a specific action on the muscarinic receptors. The involvement of apoptosis, necrosis and cleaved caspase-3 were evaluated by flow cytometry. Additional experiments were undertaken via the use of Z-DEVD-FMK (DEVD, 7×10^{-9} M), a potent caspase-3 inhibitor, to confirm whether the effects of ipratropium were dependent on caspase-3 activation. Further to this, a cardiomyocyte model of oxidative stress was employed to assess ipratropium's effect on mitochondrial membrane potential and rigor contracture.

Materials and Methods

Animals

Adult male Sprague-Dawley rats (350g ± 50g body weight) were bred in-house (Coventry University, Coventry, UK). All animals resided under the same conditions, received humane care and a standard diet. Procedures were carried out in accordance with the Guidance on the Operation of the Animals (Scientific Procedures) Act 1986 (The Stationary Office, London, UK).

Experimental design and isolated perfused heart model of I/R

For the isolated perfused heart (Langendorff) model of I/R, rats were sacrificed by cervical dislocation prior to excision of the heart. The heart was immediately placed in ice cold Krebs-Heinsleit buffer (1.18×10^{-1} M NaCl, 2.5×10^{-2} M $NaHCO_3$, 4.8×10^{-3} M KCl, 1.2×10^{-3} M $MgSO_4$, 1.2×10^{-3} M KH_2PO_4, 1.2×10^{-2} M Glucose, 1.7×10^{-3} M $CaCl_2 2H_2O$) and mounted on a Langendorff apparatus for retrograde perfusion with Krebs-Heinsleit buffer (maintained at 37°C, pH 7.4 and saturated with 95% O_2 5% CO_2). Following 20 min stabilisation, ischaemia was induced via insertion of a surgical suture under the left descending coronary arteries, through which the ends were passed through a plastic tube to form a snare. The snare was tightened to induce regional ischaemia, for 35 min, after which releasing the threads initiated reperfusion. All drugs were added at the onset of, and throughout, reperfusion which lasted 120 min. To delineate the ischaemic from the non-ischaemic area, at the end of reperfusion the coronary arteries were re-ligated and the heart perfused with a 0.25% Evans blue saline solution. This enabled visual differentiation between the non-ischaemic area (which stained dark blue) and the ischaemic area, which did not stain. The hearts were immediately stored at -20°C. Once frozen, hearts were transversely sliced into 2 mm thick sections and incubated at 37°C in a phosphate buffer solution containing 1% 2,3,5-triphenyltetrazolium chloride (TTC). For preservation and development of the staining, hearts were placed in a 10% formalin solution for 4 hours before analysis. After staining, the viable tissue in the

ischaemic area appeared red (tetrazolium positive) distinguishing it from the infarct tissue which was pale and white (tetrazolium negative). The heart slices were compressed between two Perspex sheets and the viable and infarct tissue from the risk zone were traced onto acetate film. Computerised planimetry (using Image Tool as developed by the University of Texas Health Science Centre at San Antonio, UTHSCSA) allowed the percentage of infarct tissue within the ischaemic area to be calculated (I/R%).

Determination of haemodynamic function

Following mounting on the Langendorff apparatus, the left atrium was removed, allowing insertion of a fluid-filled latex balloon, inflated to constant diastolic pressure of 5-10 mmHg, into the left ventricle. A physiological pressure transducer connected to a Powerlab (AD Instruments Ltd. Chalgrove, UK) via a bridge amp facilitated the regular measurement of left ventricular developed pressure (LVDP, mmHg) and heart rate (HR, bpm). Coronary flow (CF, $ml.min^{-1}$) was measured by collecting the perfusate at regular time intervals. Measurements of all three haemodynamic parameters were taken every 5 min during stabilisation and ischaemia and every 15 min throughout reperfusion. A significant drop in LVDP and CF following the initiation of ischaemia compared with LVDP and CF at stabilisation was necessary to ensure that ischaemia had been performed appropriately (data not shown),

Rat cardiac myocyte isolation protocol

Adult rat myocytes were isolated by enzymatic digestion of the extracellular matrix (Maddock *et al.*, 2002). Briefly, following sacrifice by cervical dislocation, rat hearts were quickly excised and mounted on a modified Langendorff apparatus for retrograde perfusion with a modified Krebs-Ringer's buffer (KRB) ($2.0x10^{-2}$ M $NaHCO_3$, $1.16x10^{-1}$ M NaCl, $1.2x10^{-3}$ M KH_2PO_4, $5.4x10^{-3}$ M KCl, $4.0x10^{-4}$ M $MgSO_4.7H_2O$, $1.2x10^{-3}$ M glucose, $2.0x10^{-2}$ M taurine, $5.0x10^{-3}$ M sodium pyruvate, oxygenated with 95% O_2 5%CO_2, pH 7.4 and maintained at 37 ^oC). After 2 min perfusion the buffer was switched to KRB supplemented with 0.075%

Worthingtons Type II Collagenase and 4.4×10^{-6} M $CaCl_2$, which formed the digestion buffer. Hearts were perfused with the digestion buffer for 7 min prior to removal from the apparatus and separation of the ventricles from the atria. The ventricular tissue was then minced and further incubated in the digestion buffer until enzymatic dissociation was complete. Myocytes were filtered through a nylon mesh, washed with restoration buffer (1.16×10^{-1} M NaCl, 2.5×10^{-2} M $NaHCO_3$, 5.4×10^{-3} M KCl, 4.0×10^{-4} M $MgSO_4.7.H_2O$, 1.0×10^{-2} M glucose, 2.010^{-2} M taurine, 5×10^{-3} M sodium pyruvate, 9.0×10^{-4} M $Na_2HPO_4.12H_2O$, 1% BSA and 1% Pen-Strep, at $37\,^{\circ}C$ and pH7.4) and maintained at $37\,^{\circ}C$.

Acetylcholine assay

Choline/Acetylcholine Assay Kit (Abcam, UK) was used to determine cell lysate acetylcholine levels in myocytes under normoxic conditions and those subjected to the H/R protocol. Half the myocytes from isolations used were kept under normoxic conditions, whereas the other half was subjected to the H/R protocol. The assay was carried out in accordance with the manufacturer's instruction. The assay was conducted in the absence and presence of acetylcholinesterase in order to identify values of total and free choline to establish the concentration of acetylcholine in myocyte lysates.

MTT Cell Viability assay

Ventricular myocytes were counted and resuspended in Esumi ischaemic buffer (Esumi *et al.*, 1991), prior to 4 hours hypoxia. To initiate reoxygenation, myocytes were randomly assigned to drug treatment groups and resuspended at 1×10^5cells.ml^{-1} in drug treated restoration buffer. Reoxygenation lasted 2 hours before all wells were treated with 20µl 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (5mg.ml^{-1}, in PBS) and incubated, in the dark, for a further 2 hours. 100 µl Lysis buffer (20% SDS in 50% N-N-Dimethylformamide) was administered to all groups and further incubated on an orbital shaker, in the dark, overnight, prior to colorimetric analysis at 450 nm. The hypoxia/reoxygenation (H/R) protocol was considered to have been successful when there

was an approximate 40% drop in viability in the control myocytes in comparison with myocytes not subjected to the H/R protocol (normoxic group).

Flow cytometric analysis for cleaved caspase-3 activity and cell death assay

Following hypoxia, as outlined above, myocytes were reoxygenated in the presence of the corresponding drugs for their treatment group for 4 hours. Myocytes were transferred to labelled, 1.5 ml microfuge tubes and, following the manufacturer's (Cell Signaling Technology) instruction, stained using Caspase-3 (Asp175) with Alexa Fluor® 488 Conjugate and 10,000 myocytes per sample were analysed using the FL-1 channel. Flow cytometric analysis was carried out using a FACS Calibur™ flow cytometer. In order to carry out the cell death assay, Vybrant® Apoptosis Assay Kit #10 (Life Technologies Ltd, Paisley, UK) was implemented, according to the manufacturer's instruction. This allowed for differentiation between viable, apoptotic and necrotic myocytes on the basis of respective staining with resazurin-C_{12}, Annexin V or SYTOX® Green. Stained cells were analysed via flow cytometry whereby the different myocyte populations could be distinguished from one anothe. 10,000 myocytes were analysed per sample on the FL-1, 3 and 4 channels.

Oxidative Stress Model Protocol

In total, seven animals were used, from which between 10 and 15 myocytes were measured for each experimental group. Ventricular myocytes were plated onto laminin coated petri dishes (35 mm diameter) and incubated for ≥2 hours to allow adhesion of the cells. The supernatant, including affluent cells was aspirated and discarded. The adhered myocytes were incubated in microscopy buffer (modified Krebs-Ringer's buffer with $1.0x10^{-2}$ M HEPES and $1.2x10^{-6}$ M $CaCl_2$) containing $3x10^{-6}$ M tetramethylrhodamine methyl ester (TMRM) for 15 min. Cells were washed with microscopy buffer and randomly assigned to one of the drug treatment groups and incubated for a further 15 min with $1x10^{-9}$ M – $1x10^{-6}$ M ipratropium ± acetylcholine ($1x10^{-7}$ M) and atropine ($1x10^{-7}$ M) ± acetylcholine ($1x10^{-7}$ M). To view and analyse myocytes, dishes were placed on the stage of a Zeiss 510 CLSM confocal

microscope equipped with 20x objective lens (NA 1.3) and a heated chamber. A 585 nm long pass filter allowed detection of TMRM when viewing the cells. Recording and analysis was facilitated by use of the Zeiss software package, LSM 2.8. Laser stimulation via 543 nm emission line of HeNe laser was used to induce oxidative stress. Prior to laser stimulation, the cationic TMRM selectively localises in the negatively-charged inner-membrane of the mitochondrion in a membrane potential-dependent manner. Laser stimulation initiates photodecomposition of TMRM thus generating mitochondrial reactive oxygen species, leading to disruption of the mitochondrial membrane. Depolarisation (Dep) was measured as the time at which the TMRM started to become evenly distributed throughout the cell and is indicative of loss of mitochondrial membrane potential. Subsequent hypercontracture (Hyp) of myocytes occurs shortly afterwards. The time to both Dep and Hyp were recorded.

Data analysis

All data are expressed as the mean ± SEM. ANOVA and Fisher's protected least significant difference test were used to determine statistical differences among experimental groups, via use of SPSS Statistics 17.0. A value of $p < 0.05$ was considered to be statistically significant.

Drugs and materials

Ipratropium bromide, atropine (both Tocris bioscience, Bristol, UK) and acetylcholine chloride (SIGMA, Poole, UK) were dissolved in ddH$_2$O prior to dilution for experimental use. Z-DEVD-FMK (Tocris Cookson, Bristol, UK) was dissolved in DMSO prior to dilution for experimental use. TTC and Evans blue were both purchased from SIGMA (Poole, UK). The antibodies for Cleaved Caspase-3 (Asp175) with Alexa Fluor® 488 Conjugate was obtained from New England Biolabs (Ipswich, UK). Vybrant® Apoptosis Assay Kit #10 was purchased from Life Technologies Ltd (Paisley, UK) and Choline/Acetylcholine Assay Kit from Abcam (Bristol, UK).

Results

Effect of Ipratropium and Acetylcholine on I/R

The results from 54 successful experiments were included and are presented in Figures 1 and 2. Figure 1 shows the observations from isolated perfused heart experiments where ipratropium (1×10^{-9} – 1×10^{-6} M) was administered at reperfusion. This data showed that ipratropium increases the development of infarct in a dose-responsive manner. Infarct/Risk (%) (I/R (%)) was statistically different from control hearts (51.8 ± 3.0%) at concentrations of 1×10^{-8} M and above (Table 1, A). A submaximal concentration of ipratropium (1×10^{-7} M) was used in all subsequent experiments. The results presented in Figure 2 show the effect of acetylcholine (1×10^{-7} M) administration at reperfusion. Acetylcholine significantly reduced infarct size in comparison with the control (42.7 ± 2.9% (ACh, 1×10^{-7} M) vs. 51.8 ± 3.0% (control), p<0.05). The observed increase in infarct size due to ipratropium treatment was significantly attenuated when administered in conjunction with acetylcholine (Table 1, B). Atropine (1×10^{-7} M) administration at reperfusion was also shown to significantly increase infarct development in comparison with the control (58.64 ± 1.7% (Atr, 1×10^{-7} M) vs. 51.8 ± 3.0% (control), p<0.05), which was also significantly attenuated upon acetylcholine administration (Table 1,C).

Identification of Acetylcholine levels in isolated rat cardiac myocytes

Endogenous levels of acetylcholine in primary cultures of rat cardiac myocytes were established using an acetylcholine assay kit to identify the concentration of acetylcholine in myocyte lysates. This enabled measurement of free and total choline, allowing quantification of acetylcholine levels. Under normoxic conditions (Fig.3, A & B), levels of acetylcholine were found to be 1.7×10^{-9} M. In cell lysates from myocytes subjected to the H/R protocol (Fig.3, C & D), the concentration of acetylcholine was observed as 1.3×10^{-9} M.

Effect of Ipratropium on adult rat cardiac myocyte viability

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is reduced to purple formazan by mitochondrial dehydrogenase, and therefore provides an assay for cellular viability. Figure 4 shows percentage change in MTT reductase activity, indicative of myocyte viability, of the control in comparison with myocytes administered ipratropium or atropine (1×10^{-11} M – 1×10^{-6} M). Administration of ipratropium or atropine at the onset of reoxygenation produced a significant reduction in cell viability in a dose responsive manner (Table 3). The cell viability assay was conducted in the presence and absence of acetylcholine ± ipratropium or atropine (Figure 5). Treatment with acetylcholine significantly increased cell viability (108.0 ± 4.6%, (ACh, 1×10^{-7} M) vs. 100 ± 0.0% (control), $p < 0.05$) in comparison with the control. In the group treated concomitantly with ipratropium and acetylcholine, cell viability was significantly increased in comparison with myocytes treated with ipratropium alone (88.7 ± 4.5% (Ip + Ach) vs. 73.0 ± 2.1% (Ip, 1×10^{-7} M), $p < 0.05$). A significant decrease in myocyte viability was observed in the group treated with atropine (81.3 ± 8.3% (Atr, 1×10^{-7}) vs. 100 ± 0.0% (control), $p < 0.05$). In comparison with the group treated with both atropine and acetylcholine, there was a small increase in viability in comparison with the atropine group, however, this was statistically insignificant.

Effects of ipratropium on apoptosis and necrosis

Additional experiments were conducted to identify whether the observed ipratropium induced increase in I/R (%) and reduction in cell viability was due to apoptosis or necrosis. Viable (data not shown), necrotic and apoptotic myocytye populations were recorded via flow cytometry from cardiac myocytes exposed to ipratropium treatment (1×10^{-9} M – 1×10^{-6} M) throughout reoxygenation (Figure 6). Apoptosis and necrosis were estimated by Annexin V and SYTOX® Green positive myocytes, respectively. All concentrations of ipratropium tested, caused a significant increase in apoptosis (Annexin V positive) in comparison with the untreated control (100 ± 0.0, control vs. 169.8 ± 19.7 (1×10^{-9} M), 160.9 ± 23.3 (1×10^{-8} M), 139.9 ± 18.1 (1×10^{-7} M) and 153.1 ± 17.9 (1×10^{-6} M), all $p < 0.05$. In addition ipratropium

administration caused a significant increase in necrosis (SYTOX® Green positive) above the control group at 1×10^{-8} M, 1×10^{-7} M and 1×10^{-6} M ipratropium (100 ± 0.0, control vs. 142.6 ± 15.5 (1×10^{-8} M), 141.6 ± 23.9 (1×10^{-7} M) and 140.9 ± 17.7 (1×10^{-6} M), all $p < 0.05$).

Effect of ipratropium treatment on cleaved caspase-3 levels following simulated iscaemia/reperfusion

Having established an increase in apoptotic death, following ipratropium administration, the involvement of cleaved caspase-3 was evaluated by flow cytometry. The data are presented in Figure 7 (B). This shows that exposure of ventricular cardiac myocytes to ipratropium throughout reoxygenation is associated with an increase in levels of cleaved caspase-3 (Asp 175), an effect which was significant when ipratropium was administered at 1×10^{-7} M and 1×10^{-6} M (100 ± 0.0, control vs. 128.7 ± 5.82 (1×10^{-7} M), $p < 0.05$, 147.5 ± 12.8 (1×10^{-6} M), $p < 0.01$. Addition of acetylcholine (1×10^{-7} M) (Figure 7 (C)) significantly decreased the level of cleaved caspase-3 compared with the control (70.2 ± 10.5 vs. 100 ± 0.0 (control), $p < 0.05$). Administration of atropine also produced a significant increase in caspase-3 levels in comparison with the control (119.8 ± 6.73 vs. 100 ± 0.0 (control), $p < 0.05$). However, the co-administration of ipratropium or atropine with acetylcholine produced effects which, although showed a decrease in cleaved caspase-3, were statistically insignificant in comparison with the respective groups treated with ipratropium or atropine alone.

In addition, the involvement of caspases in myocardial cell death following ipratropium treatment was assessed in perfused hearts via the administration of Z-DEVD-FMK (DEVD, 7×10^{-9} M, a potent caspase-3 inhibitor) in conjunction with ipratropium (1×10^{-7} M) at the onset of reperfusion. These data are presented in Figure 8. Administration of DEVD produced a significant reduction in I/R (%) in comparison with the untreated control ($51.8 \pm 3.0\%$, control vs. $29.7 \pm 4.7\%$, DEVD, $p < 0.01$). As previously demonstrated, the group treated with 1×10^{-7} M ipratropium induced a significant increase in infarct/risk (%) of $75.1 \pm 4.3\%$. In the group treated with both DEVD and Ipratropium, infarct development was

significantly inhibited in comparison with both the untreated control and ipratropium (1×10^{-7} M) treatment group (36.7 ± 3.7% (DEVD + Ip), vs. 51.8 ± 3.0% and 75.1 ± 4.3% respectively, both p<0.01).

Involvement of mitochondria following ipratropium administration in a model of oxidative stress

Having identified involvement of both apoptotic and necrotic myocyte death following ipratropium administration, a myocyte model of oxidative stress was employed to determine the action of ipratropium on mitochondria. Figure 9 (A) portrays the time points which were visualised and measured (depolarisation and hypercontracture) via confocal microscopy. The administration of ipratropium (1×10^{-8} M and 1×10^{-7} M) significantly decreased the time taken for depolarisation to occur in comparison with the control group (201.2 ± 9.9 s, control vs. 173.8 ± 14.6 s (1×10^{-8} M) and 147.5 ± 17.5 s (1×10^{-7} M), p<0.05, Figure 9 (B)). The subsequent time to hypercontracture was also recorded, as presented in Figure 9 (C). As with depolarisation, there was a significant decrease in hypercontracture following ipratropium (1×10^{-8} M and 1×10^{-7} M) administration (585.3 ± 33.9 s, control vs. 460.5 ± 19.0 s (1×10^{-8} M) and 455.2 ± 15.3 s (1×10^{-7} M), p<0.05). In order to determine whether acetylcholine elicited protection via a mechanism involving mitochondria, the same protocol in the presence of acetylcholine ± ipratropium was conducted. Acetylcholine administration, alone (277.2 ± 26.1 s, p<0.01), and in conjunction with ipratropium (237.0 ± 9.4 s, p<0.05), was capable of significantly delaying time to depolarisation in comparison with the control (Figure 9 (D)). The subsequent time to hypercontracture was also recorded (Figure 9 (E)). These results mirrored those from depolarisation (455.1 ± 15.2 s in Ip, p<0.01, 572.9 ± 23.4 s in ACh, p<0.05 and 547.7 ± 12.1 s in Ip + ACh vs. 550.2 ± 1.7 s in control), with the exception that statistically there was no difference between the control and Ip + ACh treated groups.

Discussion

The major finding of this work is that ipratropium significantly exacerbates myocardial injury under *in vitro* conditions of simulated myocardial ischaemia/reperfusion and oxidative stress. This was observed as an increase in infarct size (I/R%) in the isolated perfused rat heart and as a decrease in myocyte viability in primary rat ventricular myocytes, established via a decrease in MTT reductase activity. The present study also demonstrates the ability of acetylcholine to abrogate the exacerbation of I/R injury in these two models. This accords with previous studies which have shown muscarinic activation is cytoprotective following an ischaemic insult (Qin *et al.*, 2003; Davidson *et al.*, 2006; Zhao *et al.*, 2010). Interestingly, in the current study, acetylcholine was capable of attenuating some of the observed ipratropium induced injury when ipratropium and acetylcholine were co-administered. This indicates that the inhibitory action of ipratropium, as a competitive antagonist, at the cardiac muscarinic receptors is responsible for eliciting the observed injury.

In the current study the presence of acetylcholine in *in vitro* tissue was confirmed, as shown by Doležal and Tuček (1983), via use of the acetylcholine assay. However, ipratropium administration was sufficient to significantly increase myocardial injury in the absence of exogenous acetylcholine ($1x10^{-7}$ M). Previous work has shown that acetylcholine protects the myocardium following I/R (Li *et al.*, 2011) by permitting activation of reperfusion injury salvage kinase (RISK) signalling cascades (Shanmuganathan *et al.*, 2005; Tsang and Yellon, 2005), maintenance of gap junctional integrity (Yue *et al.*, 2006; Zhao *et al.*, 2010) and prevention of apoptosis (Kim *et al.*, 2008; Liu *et al.*, 2011). This study supports the protective properties of acetylcholine and postulates that the administration of ipratropium is sufficient to compete with endogenous acetylcholine, which accounts for the observed inhibition of acetylcholine induced myocardial protection following I/R.

The parallel studies using atropine have generated supporting evidence for muscarinic involvement as through this work increases in infarct development and cleaved capase-3

levels as well as decreased myocyte viability were observed with atropine administration. It was also demonstrated that acetylcholine was capable of compensating for some of the observed atropine induced injury when administered in conjunction with atropine. Ipratropium is synthetic atropine derivative (Wood *et al*., 1995), and both drugs are non-selective muscarinic antagonists. The ability for both atropine and ipratropium to exacerbate myocardial injury in the experimental models presented here implies a common mechanism of action of the two drugs which reinforces the theory that muscarinic signalling may be involved in ipratropium induced exacerbation of myocardial injury.

During reperfusion, myocyte loss can occur through both apoptosis and necrosis (Kung *et al*., 2011). Administration of ipratropium was associated with an increase in both necrotic and apoptotic myocyte death in comparison with the untreated group.

Furthermore, it was demonstrated that ipratropium administration, at the onset of reoxygenation in isolated rat cardiac myocytes, was associated with an increase in levels of cleaved caspase-3. Caspase-3 is an essential effector caspase in the caspase-dependent apoptotic signalling cascade. Caspases exist as zymogens but are only cleaved to form active enzymes following the onset of apoptosis. Significant increases in cleaved caspase-3 levels were observed following ipratropium administration, thereby indicating that ipratropium exacerbation of myocardial injury involves a caspase dependent apoptotic component. This was further supported by the isolated perfused heart studies using ipratropium and Z-DEVD-FMK (DEVD), an irreversible inhibitor of caspase-3 activation. These data identified that the observed ipratropium induced increase in I/R (%) was abrogated when administered in conjunction with DEVD.

The levels of caspase-3 in the acetylcholine treatment group were significantly lower than in the control group, indicating that acetylcholine myocardial protection is, in part, due to a reduction in apoptosis. This is also supported by other studies where acetylcholine induced cardioprotection has been shown to protect via regulation of apoptosis (Kim *et al*., 2008; Liu

et al., 2011) and promotes the hypothesis that caspase activation is necessary in order for ipratropium to exacerbate myocardial injury following I/R. However, from other results in this study, there is also a necrotic component to the observed ipratropium induced myocyte loss.

The conditions of oxidative stress, as associated with I/R, lead to loss of mitochondrial membrane potential (Hausenloy *et al.*, 2002; Halestrap and Pasdois, 2009). This causes primary necrosis due to ATP depletion (Yellon and Hausenloy, 2007) which occurs via the resultant collapse of mitochondrial function after disturbance of the mitochondrial membrane potential (Crompton, 1999). Cell death via apoptosis can also occur due to outer mitochondrial membrane rupture (Hausenloy *et al.*, 2002; Halestrap and Pasdois, 2009). The oxidative stress model used in this study measured depolarisation, indicative of loss of mitochondrial membrane potential, and also subsequent hypercontracture, rigour contracture of myocytes. The results from this model showed that administration of ipratropium exacerbated injury following free radical generation as quantified by a reduction in time to both depolarisation and hypercontracture. This work provides further evidence that ipratropium induces injury via involvement of the mitochondria. Given that Sun *et al.* (2010) indicated that acetylcholine may protect myocardial myocytes from I/R injury via a mechanism which stabilises mitochondrial membrane potential (Sun *et al.*, 2010), it is possible that the inhibitory effect of ipratropium at muscarinic receptors may prevent this action which could lead to the observed exacerbation of myocardial injury.

In addition, the ability for acetylcholine to significantly increase times to depolarisation and hypercontracture indicates that acetylcholine exerts a protective effect via preservation of mitochondrial membrane integrity. Indeed, it has been shown that acetylcholine elicits a cardioprotective response in I/R via a mitochondrial ATP-sensitive potassium channel which assists in the prevention of necrosis and apoptosis via maintenance of mitochondrial membrane potential (Sun *et al.*, 2010). The protective properties of acetylcholine observed within this study support these observations; as such, the work presented here implies that ipratropium is potentially capable of inhibiting the protective properties of endogenous

acetylcholine. In this study, it was demonstrated that endogenous levels of acetylcholine were present in the *in vitro* models used. However, exogenous elevation of acetylcholine was capable of attenuating the ipratropium induced injury. This indicates that the action of ipratropium as a competitive antagonist at cardiac muscarinic receptors may play a role in the observed ipratropum induced myocardial injury.

Despite all treatments groups showing significance in comparison with the control group at depolarisation, the differences between treatment groups and control were not as marked when hypercontracture was measured. Hypercontracture is rigor contracture of myocytes due to lack of ATP, however, depolarisation is indicative of loss of mitochondrial membrane integrity which may be considered a "point of no return" such that, once this event has occurred, cells are committed to death (Baines, 2009). Therefore, although it is interesting that the times to hypercontracture vary less among groups, it is the time to depolarisation which has determined the fate of the myocytes and ostensibly this is more important as following depolarisation the mitochondrion will no longer be capable of producing its own ATP, and quickly deplete ATP levels from surrounding, still functioning mitochondria, initiating the first, irreversible steps towards a cellular death pathway. In a sense, the time to hypercontracture is irrelevant as myocyte death following depolarisation is inevitable.

These data suggest that ipratropium is capable of eliciting myocardial injury following ischaemia/reperfusion by a mechanism which involves disruption of mitochondrial membrane potential as well as caspase-dependent apoptosis.

As muscarinic activation has been shown to protect via the RISK pathway through a mechanism which maintains mitochondrial membrane potential, it is possible that the antagonist action of ipratropium on muscarinic receptors in the heart may cause involvement of phosphoinositide 3-kinase (PI(3)K) and extracellular signal-regulated kinases 1/2 (Erk1/2). This may cause deregulation of RISK pathway signalling, however the exact mechanism of the pathway remains to be elucidated.

From a clinical perspective, further investigation is required to identify whether there is a real risk for COPD patients with underlying IHD who are receiving anticholinergic drugs. Although modern anticholinergics, such as ipratropium, have been favoured due to limited bioavailability, 90% of inhaled ipratropium is estimated to be swallowed post administration (Cugell, 1986) and the bioavailability is postulated to be approximately 6.9% (Ensing *et al.*, 1989). This indicates a potential for ipratropium to antagonise muscarinic receptors outside the pulmonary system, for example, in the myocardium. Further to this, around 50% of all COPD deaths are due to adverse cardiovascular outcomes, with MI remaining the most common cause of death in COPD patients (Zielinski *et al.*, 1997; Huertas and Palange, 2011). It is therefore imperative that further studies are conducted to ascertain whether our observations have a clinical implication for COPD patients with underlying IHD.

To summarise, this work is the first to identify cardiotoxic effects of ipratropium in a non-clinical setting of myocardial ischaemia/reperfusion. It has been ascertained that a) ipratropium exerts its toxic effects via disruption of mitochondrial membrane potential, accompanied by an increase in caspase-3 activity and b) the observed ipratropium induced toxicity is abrogated by acetylcholine, indicating the observed injury is via potential involvement of muscarinic receptor intracellular signalling mechanisms, leading to increased myocyte loss via both apoptosis and necrosis.
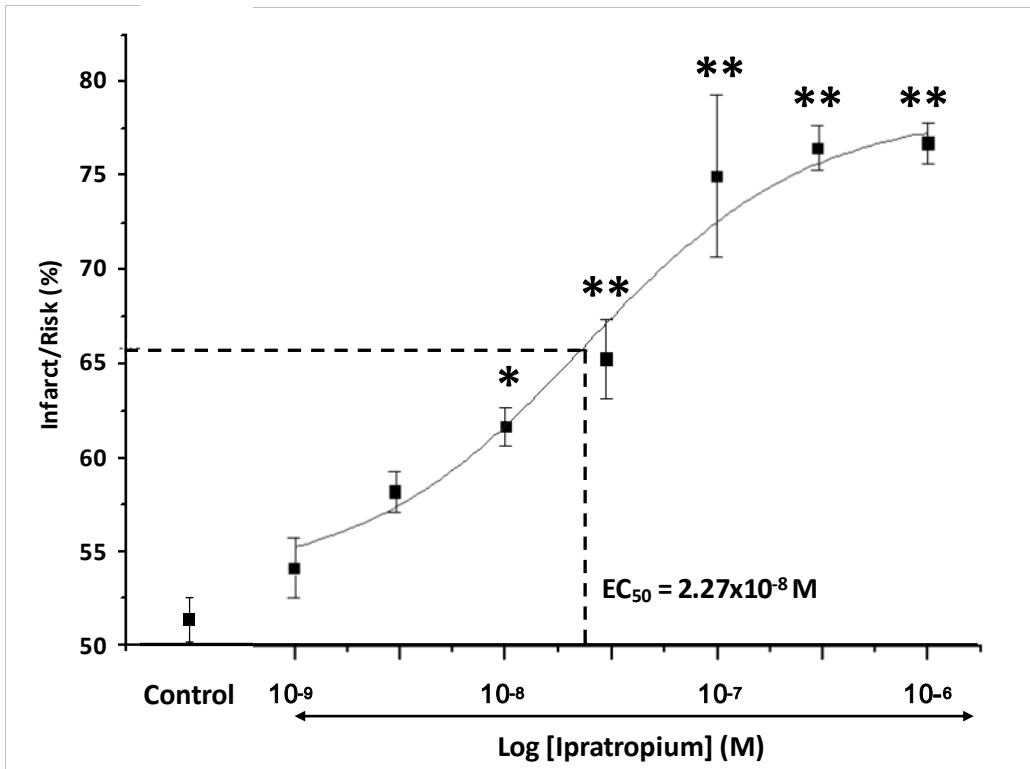
Figure 1 Infarct development in the risk zone after administration of different concentrations of ipratropium (1×10^{-9} M – 1×10^{-6} M) when administered at the onset of, and throughout, reperfusion. Results are presented as infarct/risk ratio (Infarct/Risk %). Expressed as arithmetic mean ± SEM. *$p<0.05$ and **$p<0.01$ vs. control (51.8 ± 3.0%) n=6.
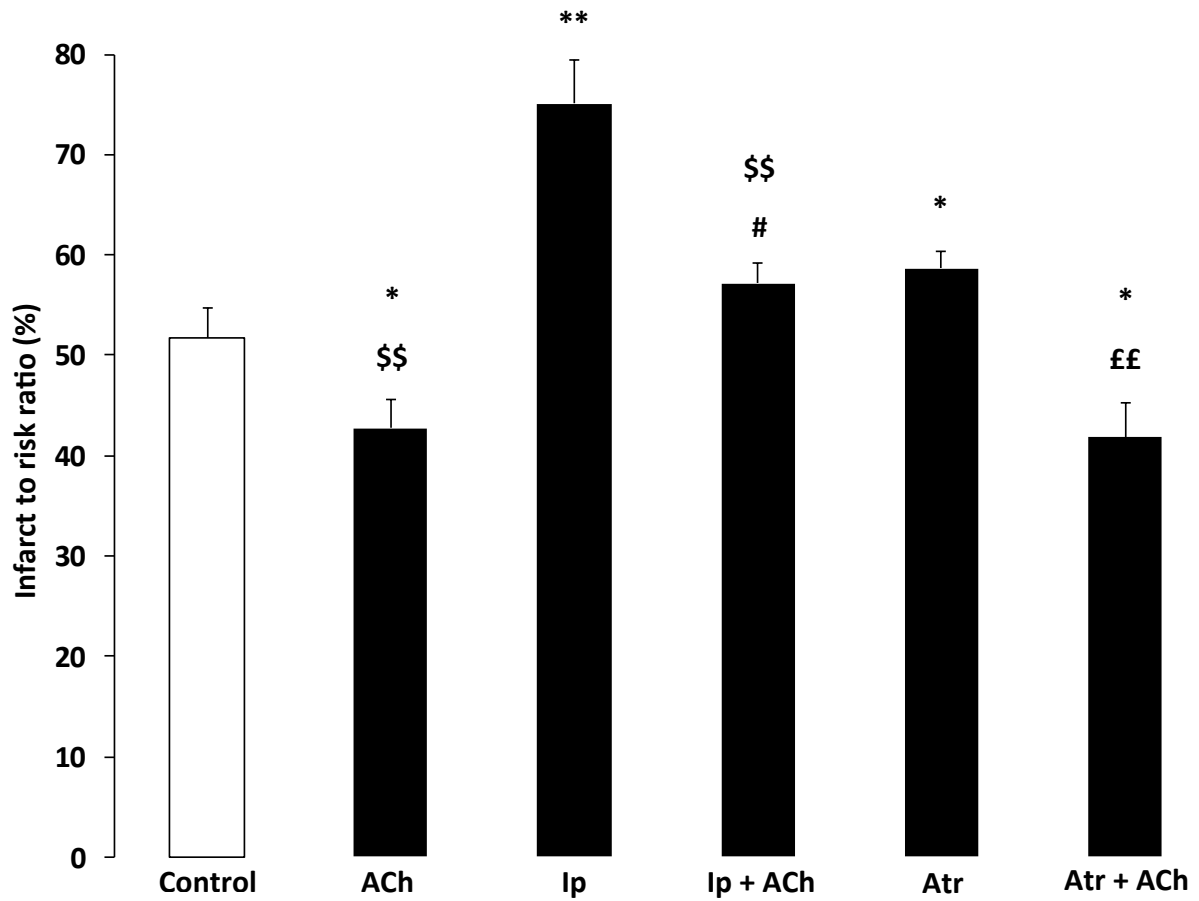
Figure 2 The effect of 1×10^{-7} M acetylcholine (ACh) administration at reperfusion on ipratropium (1×10^{-7} M) and atropine (1×10^{-7} M) induced myocardial injury in the isolated perfused rat heart. Results are presented as infarct/risk ratio (I/R%). Mean ± SEM, n=6, *$p<0.05$, **$p<0.01$ vs. control, $$$p<0.01$ vs. ipratropium, #$p<0.05$ vs. Ach, ££$p<0.01$ vs. atropine .
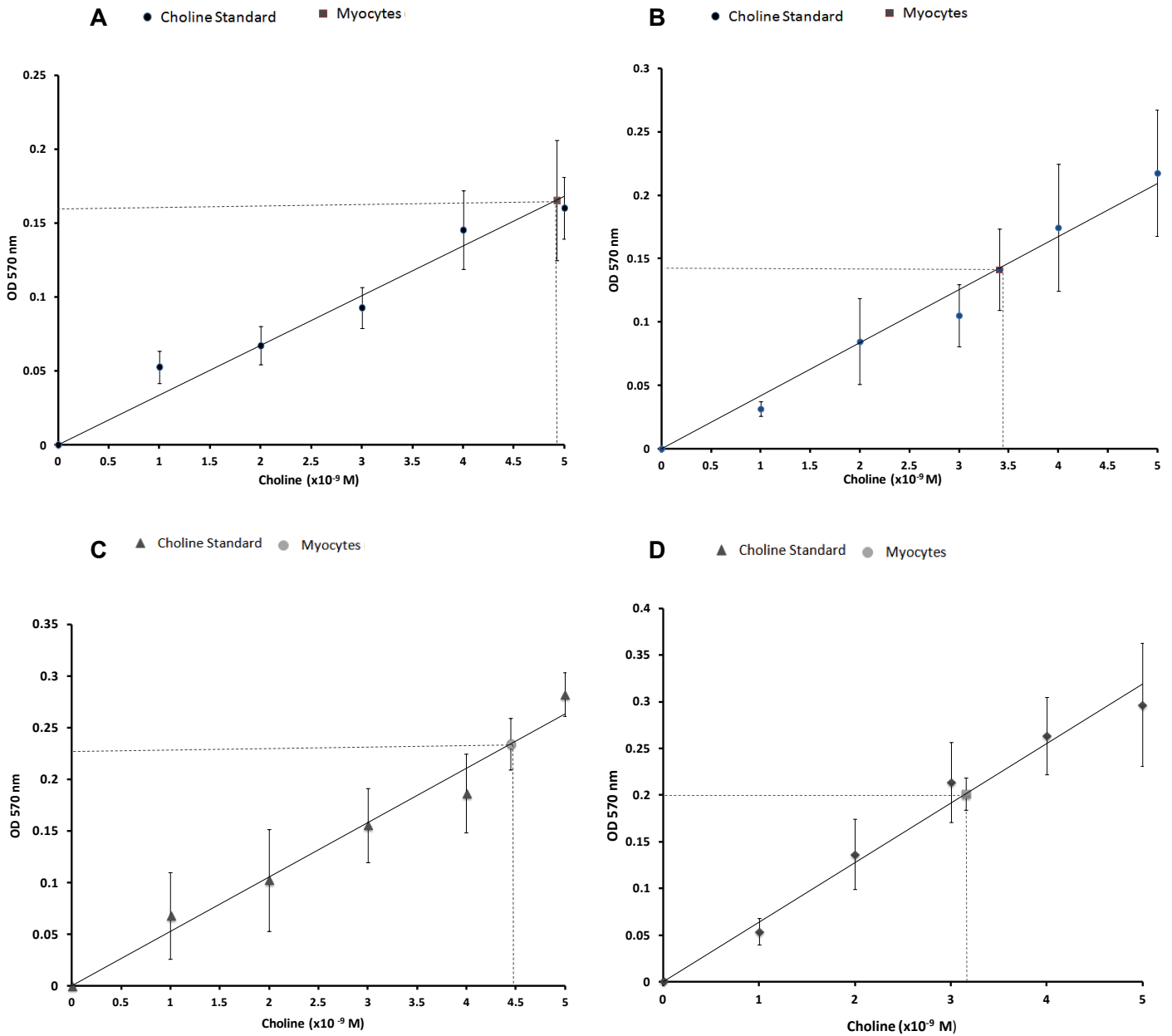
Figure 3 Endogenous levels of acetylcholine as determined by choline/acetylcholine assay, myocytes at 1×10^6 cells.ml^{-1}. Panels A and B Levels of free choline (A, 4.92×10^{-9} M $\pm$ 0.04) and total choline (B, 3.41×10^{-9} M $\pm$ 0.03) in normoxic, untreated adult rat ventricular myocytes. Panels C and D: Levels of free choline (C, 4.49×10^{-9} M $\pm$ 0.03) and total choline (D, 3.16×10^{-9} M $\pm$ 0.02) in untreated adult rat ventricular myocytes subjected to hypoxia/reoxygenation protocol. Expressed as arithmetic mean $\pm$ SEM, n=5.
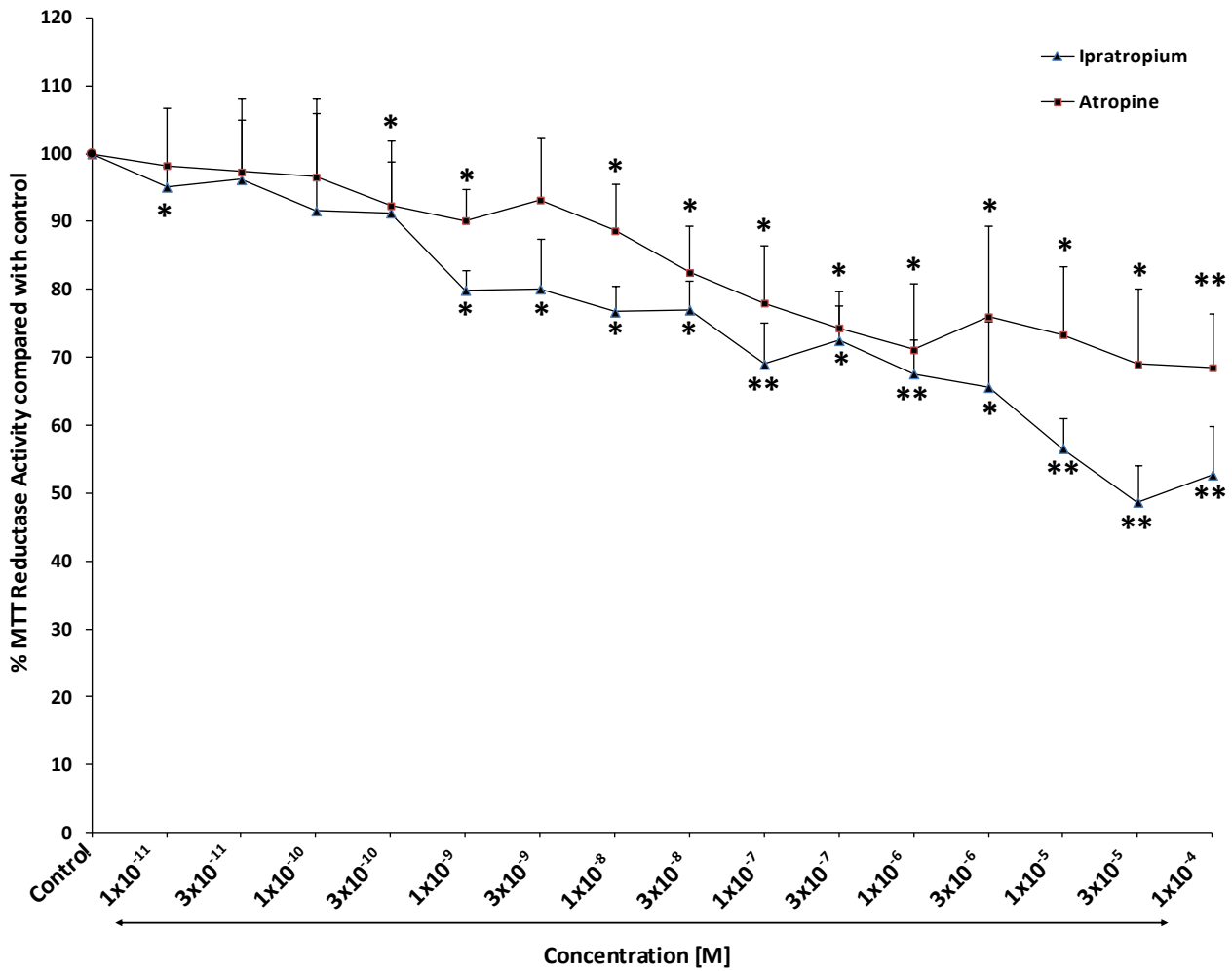
Figure 4 Assessment of myocyte viability via changes in MTT reductase activity in isolated rat ventricular myocytes following hypoxia/reoxygenation. Administration of ipratropium or atropine (1×10^{-11} M – 1×10^{-4} M) occurred at the onset of, and throughout, reoxygenation. All groups were subjected to hypoxia/reoxygenation protocol. Values are mean ± SEM, n=6. *$p < 0.05$, **$p < 0.01$ vs. control, n=6.
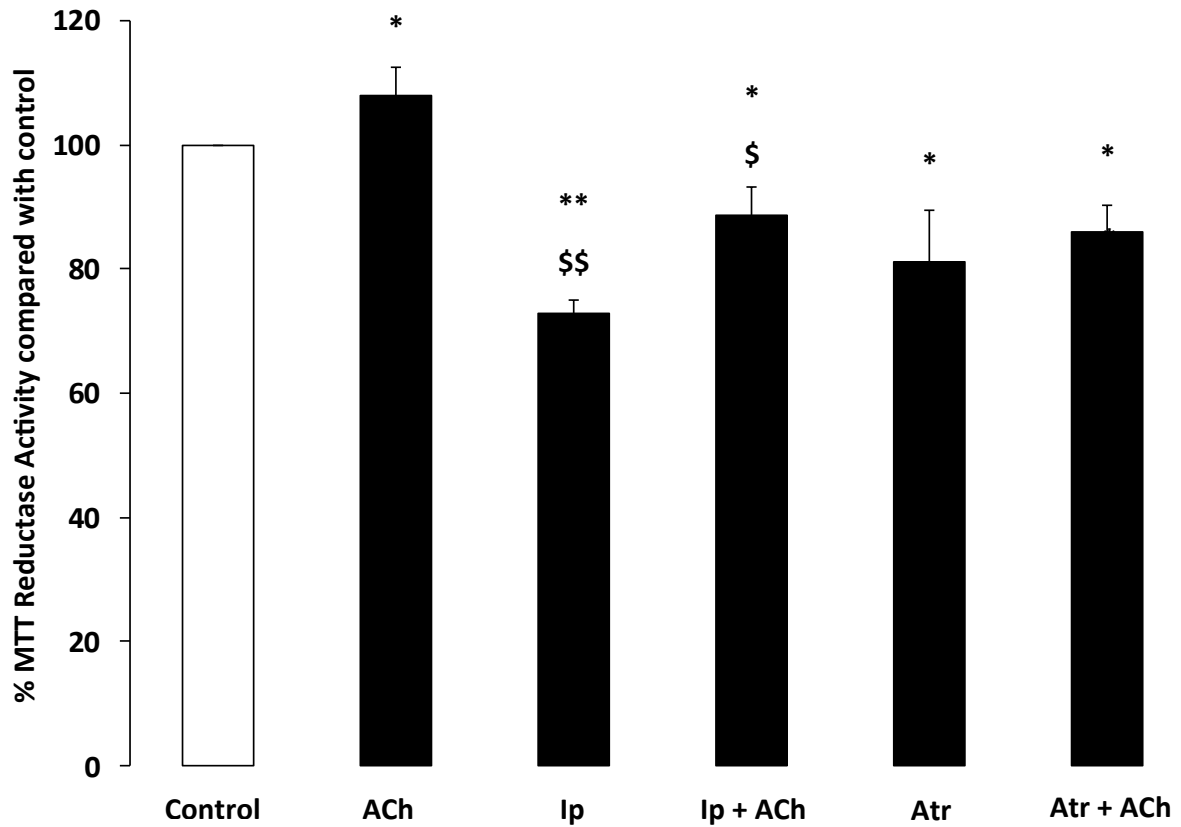
Figure 5 Effect of acetylcholine ± ipratropium on MTT reductase activity in isolated rat ventricular myocytes following hypoxia/reoxygenation, normalised as a percentage of the untreated control. Administration of ipratropium (Ip 1×10^{-7} M), atropine (Atr, 1×10^{-7} M) and acetylcholine (ACh, 1×10^{-7} M) occurred at the onset of, and throughout, reoxygenation. All drug treated groups were subjected to hypoxia/reoxygenation protocol. Values are mean ± SEM, n=6. *p<0.05, **p<0.01 vs. control, $p<0.05, $$p<0.01 vs. ACh (1×10^{-7} M).
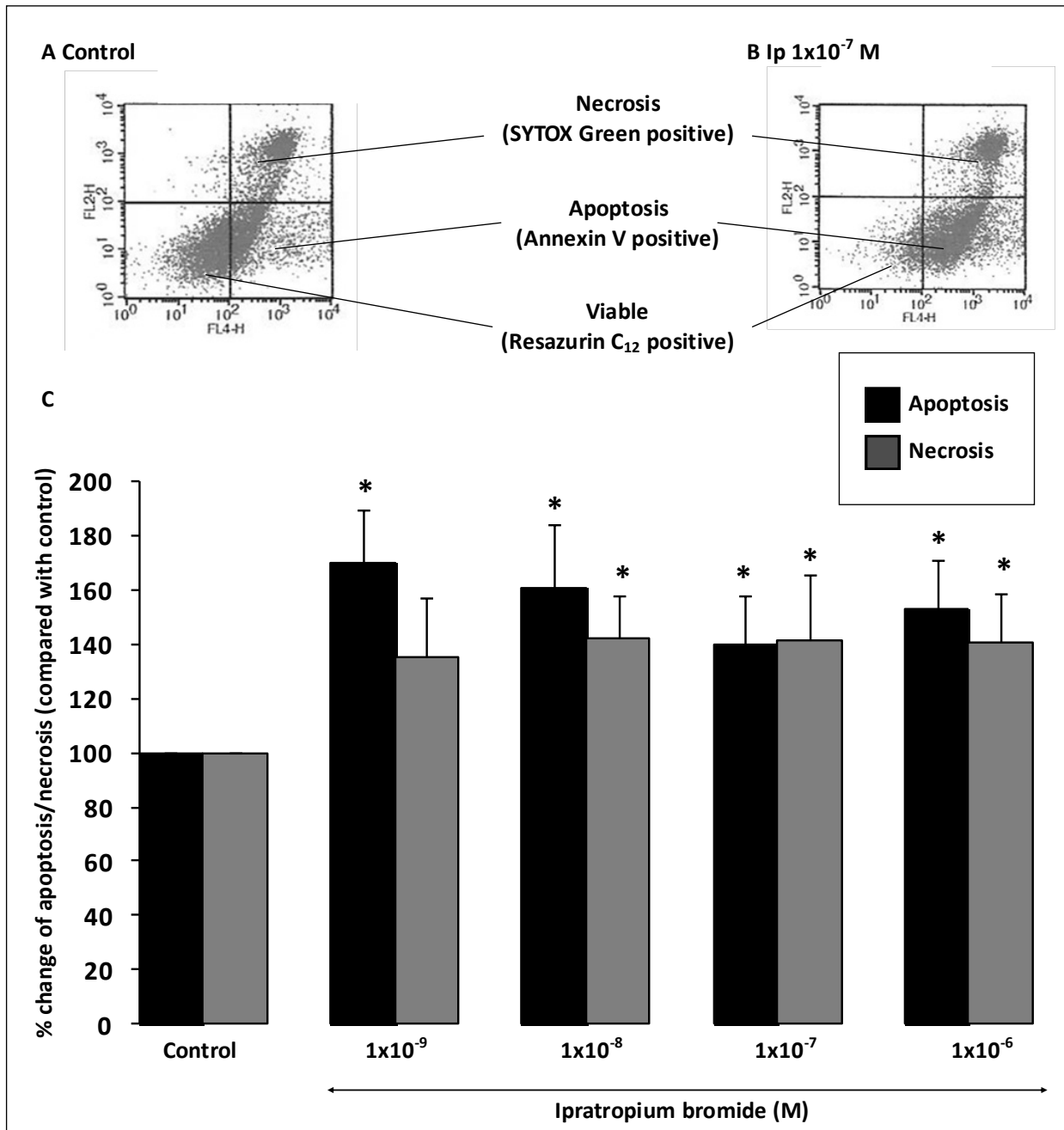
Figure 6 Assessment of apoptosis and necrosis in adult rat ventricular cardiac myocytes following H/R protocol and ipratropium treatment. A and B, original representative flow cytometric scatter graphs presenting myocytes subjected to hypoxia and reoxygenation in the absence (A) and presence (B) of ipratropium to indicate the differentiation between necrotic, apoptotic and viable myocytes. C. Depicts normalised data for apoptotic and necrotic myocyte populations expressed as percentage of untreated control. *p<0.05 in comparison with respective untreated control, values are mean ± SEM (10,000 myocytes recorded per group, n=10)
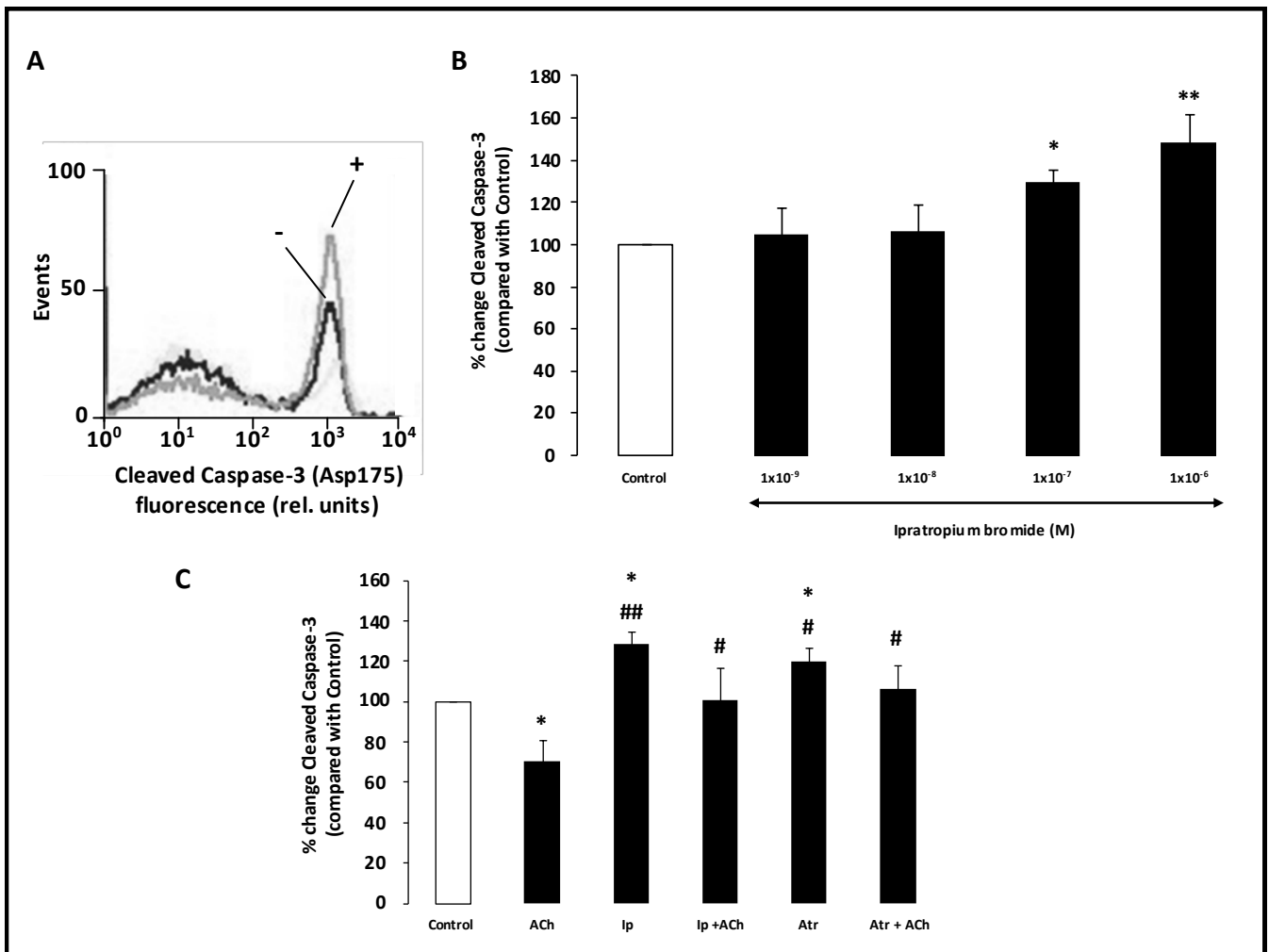
Figure 7 Effect of ipratropium (Ip), atropine (Atr) and acetylcholine (Ach) on cleaved caspase-3 levels in ventricular myocytes, following hypoxia and reoxygenation. Ipratropium, atropine and acetylcholine administered throughout reoxygenation. A. Original histogram depicting cleaved caspase-3 fluroescence (Alexa Fluor 488® conjugate) in myocytes following 2 hours hypoxia and exposure to reoxygenation without (-) and with (+) 1×10^{-7} M ipratropium. B. Effect of ipratropium (1×10^{-9} M – 1×10^{-6} M) on caspase-3 levels of normalised histogram data expressed as percentage of untreated control (white bar). C. Effect of acetylcholine (ACh, 1×10^{-7} M) ± ipratropium (Ip, 1×10^{-7} M) and atropine (Atr, 1×10^{-7} M) on levels of cleaved caspase-3 activity in isolated rat ventricular myocytes following hypoxia/reoxygenation, as determined by flow cytometric analysis. Expressed as arithmetic means, normalised to untreated control, ± SEM, 10,000 myocytes recorded per group,n=6), *$p<0.05$, **$p<0.01$ vs. respective control. C, #$p<0.05$, ##$p<0.01$ vs. ACh (1×10^{-7} M).
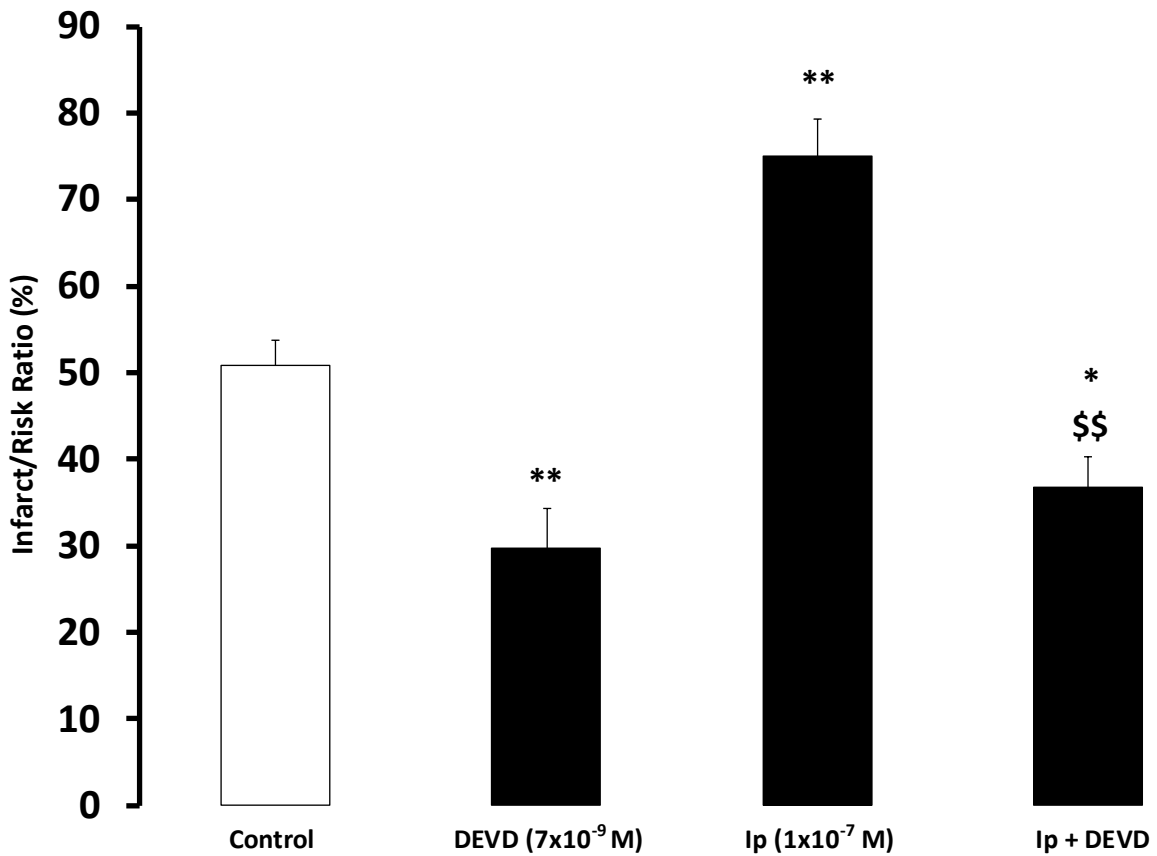
Figure 8 Infarct development in the risk zone following Z-DEVD-FMK (7×10^{-9} M) treatment ± ipratropium (1×10^{-7} M) in isolated perfused rat heart. DEVD and ipratropium both administered at the onset of reperfusion. Results are presented as infarct/risk (%).* $p<0.05$ (36.7 ± 3.7 (DEVD + ipratropium) and ** $p<0.01$ (29.7 ± 4.7 (DEVD) and 75.1 ± 4.3 (ipratropium) vs. control (51.8 ± 3.0, white bar). $$ $p<0.01$ (36.7 ± 3.7 (DEVD + ipratropium) vs. 75.1 ± 4.3 (ipratropium)). There is no significance between the DEVD and DEVD + ipratropium groups, Mean ± SEM, n=6.

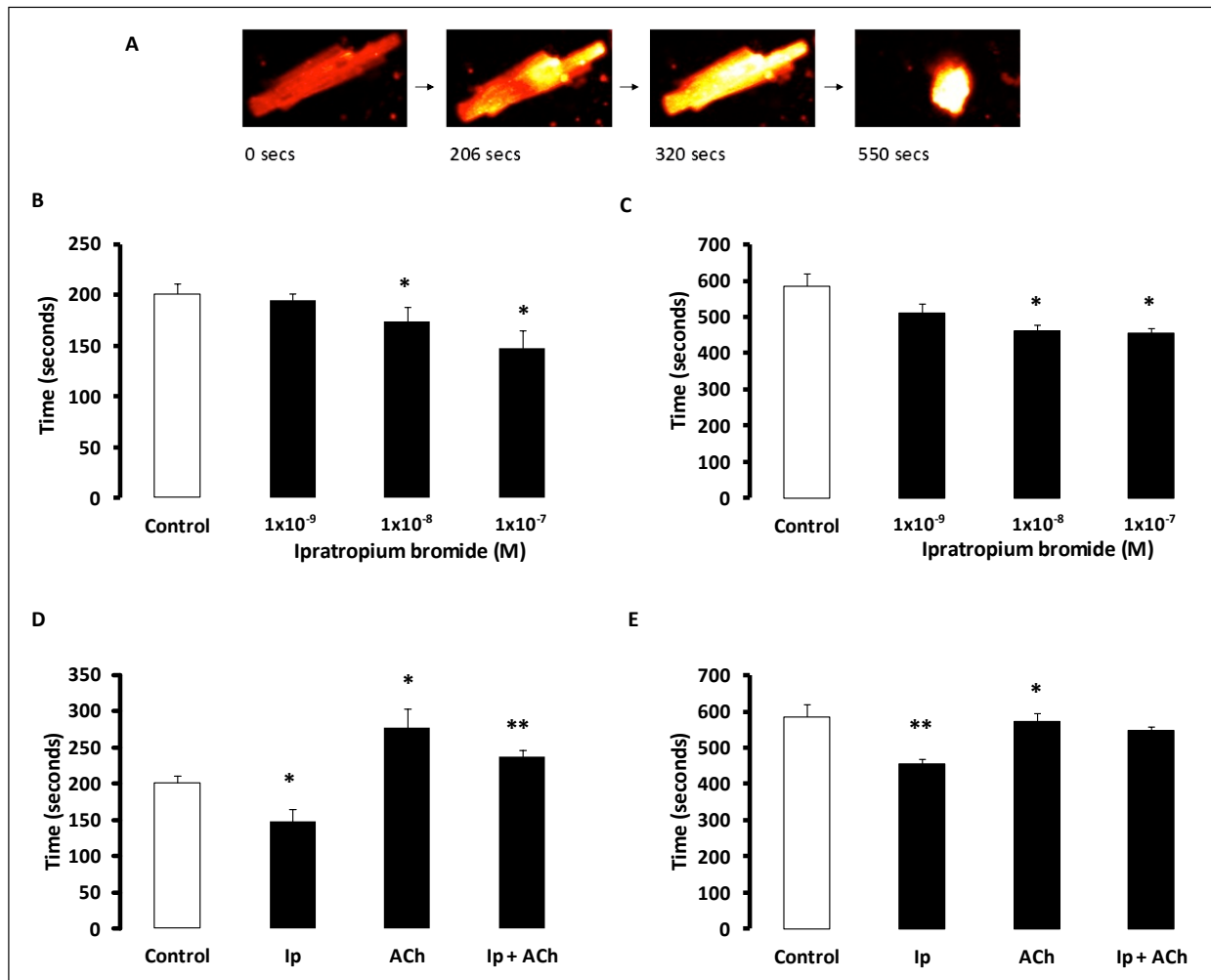**Figure 9** Determination of myocyte depolarisation and hypercontracture as established using TMRM loaded cells subjected to laser stimulated oxidative stress generation. Time points measured are schematically represented (A) the time to depolarisation, 2nd panel, (201.2 ± 9.9 secs) and hypercontracture, 4th panel (585.3 ± 33.9 secs), as visualised by confocal microscopy. Effect of ipratropium (1x10^{-9} M - 1x10^{-7} M) on time to depolarisation (B) and hypercontracture (C). Effect of acetylcholine (ACh, 1x10^{-7} M) ± ipratropium (Ip, 1x10^{-7} M) on time to depolarisation and hypercontracture (D and E, respectively), values are mean ± SEM, n=6 animals, with between 10 and 15 myocytes measured, per animal, for each group. *p<0.05, ** p<0.01 vs. respective control (white bars).

Group	Infarct/Risk (%) (Mean ± SEM)
Control	51.8 ± 3.0
(A)	
Ipratropium bromide:	
1×10^{-9} M	54.2 ± 1.6
3×10^{-9} M	58.3 ± 1.1
1×10^{-8} M	61.7 ± 1.9 *
3×10^{-8} M	65.3 ± 2.1 **
1×10^{-7} M	75.1 ± 4.3 **
3×10^{-7} M	76.6 ± 1.2 **
1×10^{-6} M	76.8 ± 1.1 **
(B)	
ACh (1×10^{-7} M)	42.7 ± 2.9 * $$
Ip + ACh (both 1×10^{-7} M)	57.2 ± 2.0 $$
(C)	
Atr (1×10^{-7} M)	58.6 ± 1.7 *
Atr + ACh (both 1×10^{-7} M)	41.8 ± 3.5 * ££

Table 1 Values for infarct development as expressed graphically in Figures 1 and 3. (A) I/R (%) values for isolated perfused rat hearts treated with ipratropium (1×10^{-9} M – 1×10^{-6} M). Mean ± SEM, *$p<0.05$,**$p<0.01$ vs. control. (B) I/R (%) values for isolated perfused rat hearts treated with acetylcholine (1×10^{-7} M) ± ipratropium (1×10^{-7} M). Mean ± SEM, *$p<0.05$, $$ $p<0.01$ vs. ipratropium (1×10^{-7} M), ££$p<0.01$ vs. atropine, n=6 for all groups.

Group	MTT Reductase activity c/w Control (%) (Mean ± SEM)	
Control	100 ± 0.0	
	Ipratropium bromide:	Atropine:
1×10^{-11} M	95.1 ± 2.9 *	98.3 ± 8.6
3×10^{-11} M	96.2 ± 12.0	97.3 ± 7.8
1×10^{-10} M	91.6 ± 14.4	96.6 ± 11.6
3×10^{-10} M	91.3 ± 10.7	92.3 ± 6.5 *
1×10^{-9} M	79.9 ± 3.0 *	90.2 ± 4.6 *
3×10^{-9} M	80.1 ± 7.3 *	93.2 ± 9.1
1×10^{-8} M	76.7 ± 3.8 *	88.7 ± 6.9 *
3×10^{-8} M	77.0 ± 4.4 *	82.5 ± 6.9 *
1×10^{-7} M	69.0 ± 6.2 **	78.0 ± 8.5 *
3×10^{-7} M	72.5 ± 5.1 *	74.4 ± 5.5 *
1×10^{-6} M	67.6 ± 5.0 **	71.1 ± 9.7 *
3×10^{-6} M	65.6 ± 9.8 *	76.0 ± 13.5 *
1×10^{-5} M	56.6 ± 4.4 **	73.4 ± 10.2 *
3×10^{-5} M	48.7 ± 5.4 **	69.0 ± 11.2 *
1×10^{-4} M	52.7 ± 7.3 **	68.5 ± 8.0 **

Table 2 Values for the effect of ipratropium or atropine (1×10^{-11} M – 1×10^{-4} M) on MTT reductase activity as expressed graphically in Figure 4. Values expressed as a percentage of MTT reductase activity in the untreated control group. Mean ± SEM, *p<0.05, **p<0.01 vs. control, n=6 for all groups.

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