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Involvement of different α_2 adrenoceptor subtypes in effects of dexmedetomidine on porcine coronary artery

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Objective: Although the α_2-adrenoceptor receptor (α_2-AR) agonist dexmedetomidine causes coronary vasoconstriction, activation of endothelial α_2-ARs is known to induce vascular relaxation. Here, we used functional studies and molecular techniques to explore the involvement of different α_2-AR subtypes (α_{2A}-AR, α_{2B}-AR α_{2C}-AR) in the effects of dexmedetomidine on the coronary artery. *Methods*: After precontraction with prostaglandin F_{2a}, changes in tension caused by dexmedetomidine, in the presence or absence of different antagonists, were studied in isolated right porcine coronary rings in organ chambers. The presence or absence of different subtypes of α_2-ARs in right coronary arteries and in blocks of subendocardial myocardium containing coronary resistance arterioles was investigated using immunofluorescence, western blotting and reverse transcription-polymerase chain reaction (RT-PCR). *Results*: Dexmedetomidine caused relaxation of isolated large porcine coronary arteries, which was attenuated by rauwolscine (a non-selective α_2-AR antagonist), BRL44408 (an α_{2A}-AR antagonist) and ARC239 (an α_{2B}-AR antagonist), and abolished by L-NAME (a nitric oxide synthase inhibitor) and pertussis toxin (a G_i protein inhibitor), and by removal of the endothelium. Although the relaxation could also be inhibited by MK912 (an α_{2C}-AR antagonist), the pattern of inhibition suggested that it was due to a non-selective effect. The presence of α_{2A}-AR in the endothelium of the right coronary artery, of α_{2B}-AR in both the endothelium and vascular smooth muscle of the same artery, and of α_{2C}-AR in the coronary resistance arterioles was confirmed by western blotting, RT-PCR and immunofluorescence. *Conclusions*: Expression of α_2-AR subtypes is different in porcine coronary arteries of different sizes. Dexmedetomidine relaxes the large right coronary artery in an endothelium-dependent, α_{2A}-AR, α_{2B}-AR and nitric oxide-mediated manner. In contrast, α_{2C}-AR is the only type of α_2-AR in the resistance coronary arterioles and may be linked to vasoconstriction.

1. Introduction

The α_2-adrenoceptor (α_2-AR) agonist dexmedetomidine is widely used in operating rooms and intensive care units because of its sedative and analgesic effects. Emerging studies have shown that perioperative use of dexmedetomidine during both open heart and non-cardiac surgery may reduce perioperative cardiac morbidity (Biccard et al. 2008; Chi et al. 2016; Elgebaly et al. 2020; Gong et al. 2017; Wijeysundera et al. 2008). The cardioprotective effects of dexmedetomidine may involve mitochondrial K^+ channels (Raupach et al. 2020), activation of PI3K/Akt signaling (Chang et al. 2020), miR-208b-3p/Med13/Wnt/β-catenin signaling (Wang et al. 2020) and HIF-a signaling (Peng et al. 2020) pathways, as well as reduction of oxidative stress (Han et al. 2019).

Myocardial oxygen supply, which depends on normal coronary relaxation and contraction, is the determining factor for maintaining normal cardiac function. Although dexmedetomidine is cardioprotective, it is generally considered to be a coronary vasoconstrictor, which reduces coronary blood flow and myocardial perfusion, and increases coronary vascular resistance, in dogs and goats (Coughlan et al. 1992; Flacke et al. 1993; Lawrence et al. 1997; Pagel et al. 1998; Rockacrts et al. 1996). Dexmedetomidine also increases coronary vascular resistance in pigs (Jalonen et al. 1995; Kundra and Kaur 2016). Similar to the effects of dexmedetomidine in large mammals, both dexmedetomidine and another α_2-AR agonist, BHT933, reduce coronary flow in humans (Snapire et al. 2006).

The effects of dexmedetomidine on vascular tone involve interplay between the nervous system and the blood vessels themselves. Dexmedetomidine mediates sympathetic vasoconstriction (sympatholysis) by activating α_2-ARs in the central and peripheral nervous systems. In blood vessels, agonism of postsynaptic α_2-ARs on vascular smooth muscle cells may cause vasoconstriction (Talke et al. 2003), whereas agonism of postsynaptic α_2-ARs on endothelial cells may cause vasorelaxation (Figueroa et al. 2001). Dexmedetomidine is known to act on three subtypes of α_2-AR, α_{2A}-AR, α_{2B}-AR and α_{2C}-AR (Hunter et al. 1997; Moura et al. 2006). Using receptor binding and pharmacological studies, it has been shown that stimulation of α_{2D}-AR and α_{2A}-AR subtypes can induce endothelium-dependent vasorelaxation in rats (Bockman et al. 1996) and pigs (Bockman et al. 1993). In contrast, the α_{2B}-AR subtype seems to mediate vasoconstrictor responses since α_2-AR agonist-induced pressor responses are absent in α_{2B}-AR knockout mice (Link et al. 1996). In line with these studies, we previously reported a biphasic effect of dexmedetomidine on isolated rat mesenteric artery and aorta (Wong et al. 2010). At low concentrations (< 30 nM), dexmedetomidine evoked relaxation of the

mesenteric artery, followed by contraction at higher concentrations (> 30 nM). Dexmedetomidine-induced relaxation depends on nitric oxide (NO), endothelium and G_i protein, and is mediated mainly by $\alpha_{2A/D}$-ARs, and possibly α_{2B}-ARs. Contraction, on the other hand, is mediated mainly by α_{2B}-ARs and α_1-ARs and involves the actions of prostanoids. The effect of dexmedetomidine on the aorta is significantly smaller than on the mesenteric arteries, suggesting different vascular effects on different vascular beds.

2. Investigations and results

2.1. Relaxation studies

Dexmedetomidine induced a concentration-dependent relaxation of porcine coronary artery at concentrations between 100 pM and 100 nM, followed by a rebound in tension at concentrations between 100 nM and 10 µM. Treatment with L-NAME (endothelial NO synthase inhibitor) or removal of the endothelium abolished dexmedetomidine-induced relaxation (Fig. 1A). Treatment with pertussis toxin (G_i protein inhibitor, 400 ng/mL) or rauwolscine (non-selective α_2-AR antagonist, 100 nM) also led to a significant reduction in endothelium-dependent relaxation (Fig. 1B).

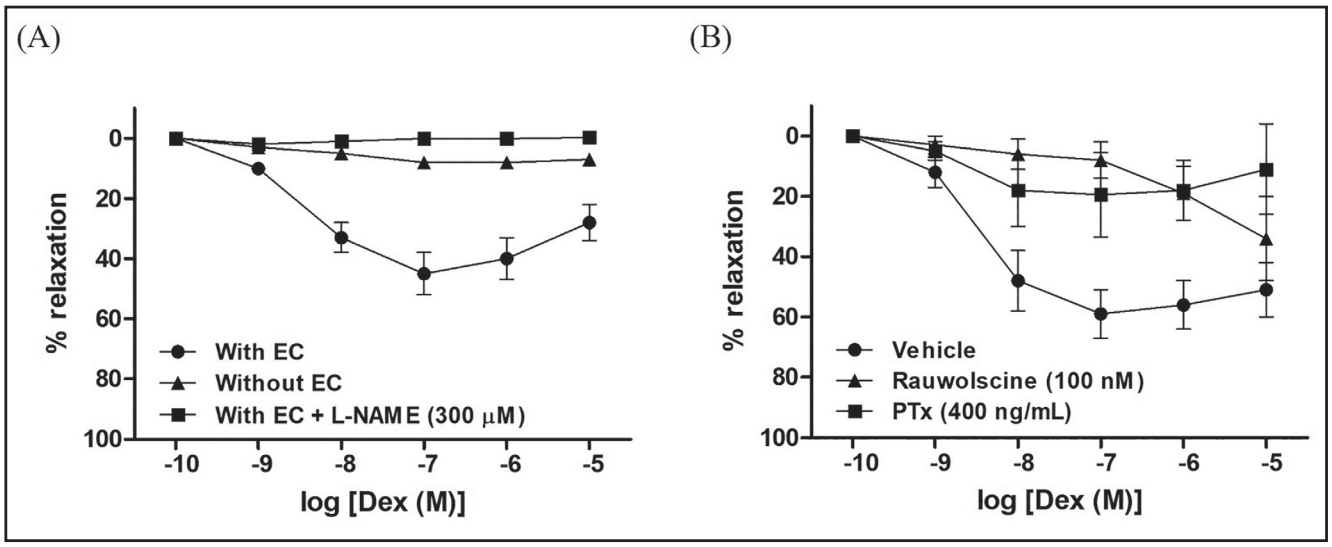

Fig. 1: Dexmedetomidine concentration-response curves in porcine right coronary rings treated with indomethacin (10 µM) and precontracted with prostaglandin $F_{2\alpha}$. (A) With intact endothelium (EC), with intact EC and treatment with L-NAME (300 µM), and with removal of endothelium ($n = 10$); (B) Treatment with rauwolscine (100 nM) or pertussis toxin (PTx, 400 ng/ mL) ($n = 10$). Data are the mean ± S.E.M. *$P < 0.05$ compared with control.

There have been very few reports describing the direct effect of dexmedetomidine on the coronary artery and the AR subtypes involved. It is important to understand the direct effect of dexmedetomidine on blood vessel cells, especially the effect on endothelial cells, since this is an important mechanism in counteracting vasoconstriction caused by the sympatholytic effect of dexmedetomidine. The objectives of this study were to evaluate the distribution of α_2-AR subtypes in large coronary arteries and small coronary arterioles, and to correlate this distribution with functional pharmacological effects.

BRL44408 ($\alpha_{2A/D}$-AR antagonist) and ARC239 (α_{2B}-AR antagonist) caused a rightward shift of the concentration-response curves compared with the control. The corresponding Schild regression line was not different from unity for BRL44408. The apparent binding affinity (pK_B) was 8.28 (95% CI, 7.86–9.19),

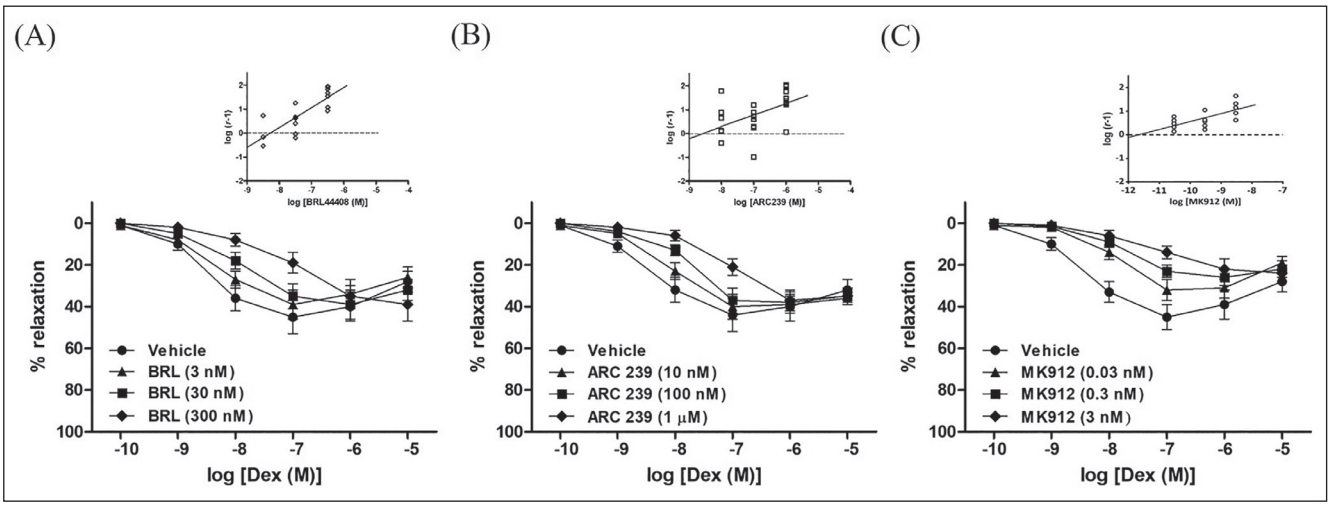

Fig. 2: Dexmedetomidine concentration-response curves in porcine right coronary rings with intact endothelium treated with indomethacin (10 µM) and precontracted with prostaglandin $F_{2\alpha}$. (A) Treatment with BRL44408 (BRL, 3–300 nM) ($n = 10$); (B) Treatment with ARC239 (10 nM to 1 µM) ($n = 10$); (C) Treatment with MK912 (0.03–3 nM) ($n = 10$). Data are the mean ± S.E.M. Insets show Schild regression analyses of concentration-response curves for BRL44408 and ARC239. *$P < 0.05$ compared with control.

which is very similar to the known binding affinity (pK_B = 8.25) of BRL44408 for cloned human α_{2A}-AR (Uhlen et al. 1994). Since the slope of the Schild regression line was different from unity for ARC239, no attempt was made to determine pK_B for ARC239 (Fig. 2A and 2B). MK912 (α_{2C}-AR antagonist) caused a rightward shift, as well as a reduction in the maximal relaxation (Fig. 2C), suggesting a mixture of competitive and non-competitive antagonism. The slope of the Schild regression line was also different from unity for MK912 so pK_B value was not determined.

2.2. *Reverse transcription-polymerase chain reaction*

RT-PCR showed that α_{2A}-AR and α_{2B}-AR mRNAs, but not α_{2C}-AR mRNA, were expressed in the right coronary artery. In contrast, expression of α_{2B}-AR and α_{2C}-AR mRNAs, but not α_{2A}-AR mRNA, was detected in subendocardial myocardium (Fig. 3).

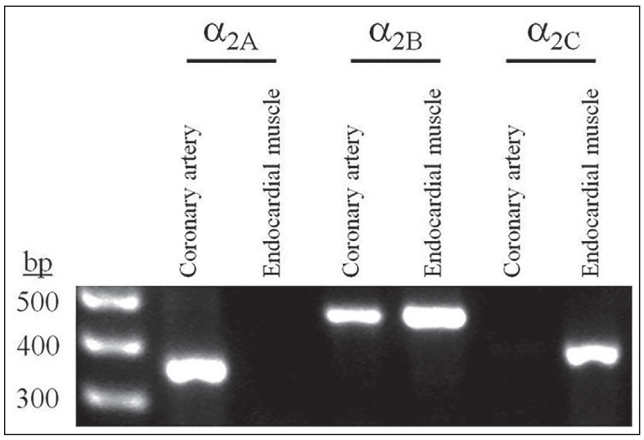

Fig. 3: RT-PCR analysis of α_2-AR mRNA in porcine right coronary arteries and subendocardial myocardium. In the coronary artery, PCR products are seen in reactions using oligonucleotide primer pairs for α_{2A}-AR and α_{2B}-AR, but not for α_{2C}-AR. In subendocardial myocardium, PCR products are seen in reactions using oligonucleotide primer pairs for α_{2B}-AR and α_{2C}-AR, but not for α_{2A}-AR. Expected sizes of amplified fragments: 363-bp (α_{2A}-AR), 459-bp (α_{2B}-AR) and 403-bp (α_{2C}-AR). DNA size markers are shown at the left.

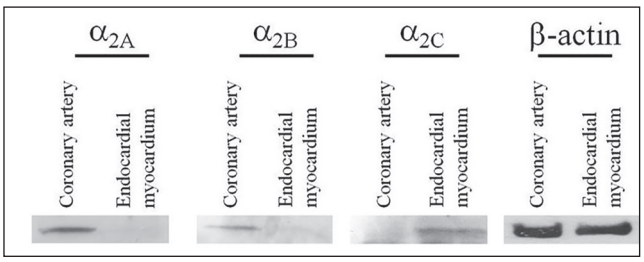

Fig. 4: Western blot analysis of α_2-AR proteins in porcine right coronary arteries and subendocardial myocardium. In the coronary arteries, α_{2A}-AR and α_{2B}-AR proteins were detected, but α_{2C}-AR was absent. In contrast, α_{2C}-AR protein was expressed in subendocardial myocardium, whereas α_{2A}-AR and α_{2B}-AR proteins were not detected. b-actin, which served as a positive control, was detected in both coronary arteries and subendocardial myocardium.

2.3. *Western blotting*

Protein bands of approximately 82 kDa were expressed in coronary arteries when using the α_{2A}-AR and α_{2B}-AR antibodies. The sizes of these protein bands were close to the reported molecular sizes of α_{2A}-AR (~72 kDa) and α_{2B}-AR (~70 kDa) in rat heart (Diebold et al. 2005). No protein expression of α_{2C}-AR could be detected in coronary arteries and no protein expression of α_{2A}-AR or α_{2B}-AR was found in the subendocardial myocardium. However, a protein band that corresponds to α_{2C}-AR protein was detected in the subendocardial myocardium (Fig. 4).

2.4. *Immunofluorescence assay*

The immunofluorescence assay showed that endothelial cells of coronary arteries were stained by the α_{2A}-AR antibody. Immunoreactive α_{2B}-AR was localized to both endothelial cells and vascular smooth muscle cells of coronary arteries, whereas no α_{2C}-AR could be detected in coronary arteries (Fig. 5). In contrast, subendocardial myocardium containing coronary arterioles was negatively stained by α_{2A}-AR and α_{2B}-AR antibodies, but immunoreactive α_{2C}-AR was detected in the endothelial cells and vascular smooth muscle cells of the arterioles (Fig. 6).

3. Discussion

In this study, we found a differential distribution of α_2-AR subtypes in porcine large coronary arteries and intramyocardial coronary resistance arterioles. The α_{2A}-AR is expressed exclusively in the

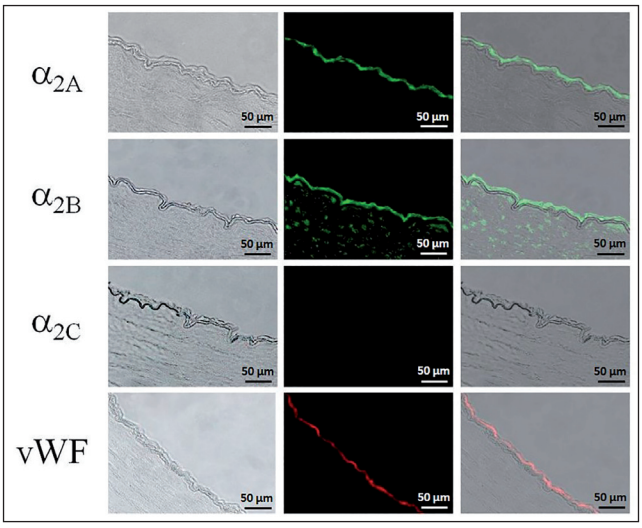

Fig. 5: Immunofluorescence staining of α_2-AR proteins in porcine right coronary arteries. Middle column: Positive immunoreactivity for α_{2A}-AR (green) was detected in endothelial cells. α_{2B}-AR (green) was localized to endothelial cells and vascular smooth muscle cells but no α_{2C}-AR was detected. Von-Willebrand factor (VWF, red) was used as a marker of endothelial cells. Left column: Phase contrast images of corresponding stained sections. Right column: Merged light micrographs and fluorescence micrographs.

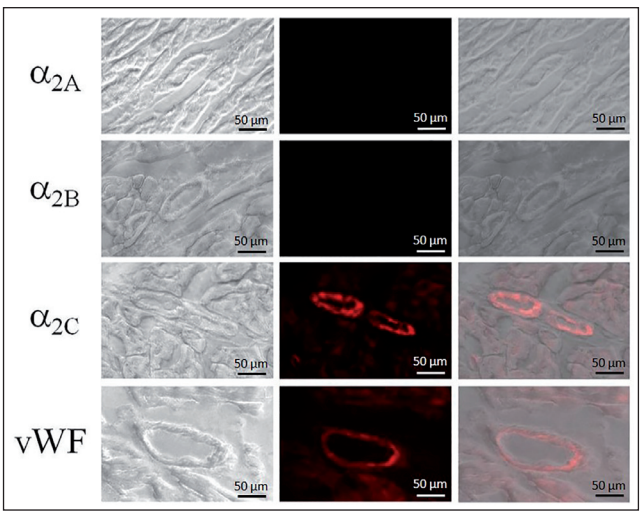

Fig. 6: Immunofluorescence staining of α_2-AR proteins in porcine subendocardial myocardium. Middle column: Positive immunoreactivity for α_{2C}-AR (red) was detected in endothelial cells and vascular smooth muscle cells, but no α_{2A}-AR or α_{2B}-AR was detected. Von-Willebrand factor (VWF, red) was used as a marker of endothelial cells. Left column: Phase contrast images of corresponding stained sections. Right column: Merged light micrographs and fluorescence micrographs.

endothelium of large coronary arteries. The α_{2B}-AR is expressed in endothelial cells and vascular smooth muscle of large coronary arteries, but the α_{2C}-AR is absent. In contrast, subendocardial myocardium does not express α_{2A}-AR. Although α_{2B}-AR mRNA was detected in the subendocardial myocardium by RT-PCR, the protein is not detectable in arterioles and cardiac muscle. This discrepancy may be due to low translation rate and/or high protein turnover in the cells, which leads to very low levels of α_{2B}-AR protein expression that are below the detection thresholds of western blot analysis and immunofluorescence staining. Expression of α_{2C}-AR mRNA and protein was observed in the subendocardial myocardium. Immunofluorescence staining showed that α_{2C}-AR is localized to endothelial cells and vascular smooth muscle cells of the coronary arterioles.

We also showed that dexmedetomidine induces endothelium-dependent relaxation of isolated porcine large coronary artery, which is likely mediated by release of NO. Our results are consistent with those of another recent study (Zhou et al. 2017), which showed that a low concentration of dexmedetomidine relaxed porcine coronary artery through an endothelium- and NO-dependent mechanism. In that study, however, the α_2-AR antagonist yohimbine failed to block the vasorelaxing effect on the coronary arteries. There are two possible explanations for this discrepancy. Firstly, in our study, we used right, large coronary arteries but the other study used left, distal coronary arteries. It has previously been reported that the vascular response to α_2-AR agonists varies in different blood vessels (Wong et al. 2010). Secondly, different α_2-AR antagonists were used in the two studies. In an earlier study, we found that the vascular effect of dexmedetomidine is different from that of the alternative α_2-AR agonists, clonidine and UK14304 (Wong et al. 2010), probably because of differences in receptor selectivity. A similar explanation can be used to explain the difference between yohimbine, which is a non-selective α_2-AR antagonist with moderate affinity for adrenergic α1, 5-HT_1 and dopamine D_2 receptors, and dexmedetomidine. In our study, using more selective antagonists, we showed that α_{2A}-AR is the major receptor subtype responsible for this relaxation. The dexmedetomidine-induced relaxation can be inhibited by pertussis toxin, suggesting the involvement of G_i-protein, which is known to be the transducing mechanism for α_{2A}-ARs. Our conclusion is also supported by estimation of antagonist affinity using the Schild regression approach. The results of immunofluorescence staining, RT-PCR and western blotting all showed that α_{2A}-AR is expressed in endothelial cells of coronary arteries. Although the α_{2B}-AR antagonist ARC239 also produced a shift in the relaxation curves for dexmedetomidine, the results of the Schild regression analysis did not suggest simple single receptor competitive antagonism. Since the α_{2B}-AR is present on both the endothelium and vascular smooth muscle in this artery, it is unclear whether this receptor subtype contributes to contraction or relaxation of coronary arteries. Development of more specific α_{2B}-AR antagonists would provide useful pharmacological tools to address this question. On the other hand, the α_{2C}-AR antagonist MK912 produced a mixed shift. Taken together with the immunofluorescence, western blotting and RT-PCR results, this may indicate that MK912 has non-selective effects on other receptor subtypes.

Although we did not perform direct functional studies on isolated coronary arterioles in this study, the absence of α_{2A}-AR and α_{2B}-AR in these arterioles suggests that dexmedetomidine may not relax these arterioles. Consistent "coronary vasoconstriction" with α_2-AR agonists, measured as reduced coronary blood flow or raised coronary resistance, has been shown by others in different species, including human, in numerous studies (Baumgart et al. 1999; Coughlan et al. 1992; Flacke et al. 1993; Indolfi et al. 1992; Lawrence et al. 1997; Pagel et al. 1998; Rockacrts et al. 1996). Dexmedetomidine thus most likely causes constriction of these arterioles via activation of α_{2C}-ARs.

Taken together, our results show that dexmedetomidine should not be considered to be simply a coronary vasodilator or a coronary vasoconstrictor. While it probably causes constriction of small coronary resistance arterioles, in agreement with the observations by other investigators of reduced coronary blood flow and increased coronary vascular resistance after administration of different α_2-AR agonists, it also relaxes large coronary arteries *via* activation of α_{2A}-AR. In patients with coronary artery disease and stenotic segments in the larger coronary arteries, constriction of small coronary resistance arterioles may not necessarily be harmful. An acute drop in coronary vascular resistance may, in fact, give rise to the phenomenon of "coronary steal" and further reduce perfusion in the ischemic regions of the myocardium (Seiler et al. 1997). On the other hand, dilatation of larger coronary arteries may improve flow characteristics in those arteries. Sudden vessel contraction, with an associated increase in flow velocity, or conversion to turbulent flow with a concomitant increase in local shear stress, may play an important role in plaque rupture (Fukumoto et al. 2008). These differential effects of dexmedetomidine on large conduit and small resistance coronary arteries may explain in part the beneficial effects of the perioperative use of different α_2-AR agonists in patients with cardiovascular risk factors, as reported in a previous meta-analysis (Wijeysundera et al. 2003).

Dexmedetomidine probably modulates the vascular tone of large coronary arteries by releasing NO from the endothelium. Although we did not directly measure NO release in this study, the abolition of relaxation after treatment with L-NAME strongly suggests that this is the case. Endothelial NO release is known to confer advantages other than modulation of vascular tone (Kawai 1994; Vanhoutte 2003). For instance, endothelial NO plays an important role in reducing excessive platelet activation, particularly at atheromatous plaques (Freedman et al. 1998; Radomski et al. 1987; Roberts et al. 2008; Schafer and Bauersachs 2008; Schafer et al. 2004). NO release is also known to favorably modulate the inflammatory response, which may also reduce the expression of thrombogenic mediators around a plaque (Tziros and Freeman 2006; Vanhoutte 2003). With probable NO release in coronary arteries following dexmedetomidine administration, these effects may further augment the beneficial effects of dexmedetomidine in patients with coronary artery disease.

Dexmedetomidine exerts its anesthetic effects via α_{2A}-ARs in the central nervous system (Hunter et al. 1997; Nelson et al. 2003). Since, as we have shown here, release of NO and coronary dilatation are also mediated by α_{2A}-ARs, more subtype-selective α_2-AR agonist anesthetics, particularly those with predominant α_{2A}-AR selectivity, may be superior to agonists that are not subtype-selective when used in patients with high cardiovascular risk.

In conclusion, we demonstrated a differential distribution of α_2-AR subtypes in the coronary circulation, and showed that dexmedetomidine dilates large coronary arteries *via* activation of α_{2A}-ARs and subsequent release of NO. Dexmedetomidine probably constricts small resistance coronary arterioles *via* activation of α_{2C}-ARs.

4. Experimental

4.1. Drugs and chemicals

Bradykinin, prostaglandin $F_{2\alpha}$, rauwolscine, BRL44408 (2-[2H-(1-methyl-1,3-dihydroisoindole)methyl]-4,5-dihydroimidazole maleate), indomethacin, MK912 (L-657,743, 2S-trans-1,3,4,5′,6,6′,7,12b-octahydro-1′,3′-dimethyl-spiro[2H-benzofuro[2,3-a]quinolizine-2,4′(1′H)-pyrimidin]-2′(3′H)-one hydrochloride hydrate), N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME) and protease inhibitor cocktail were purchased from Sigma-Aldrich (St. Louis, MO, USA). ARC239 (2-[2-(4-(2-Methoxyphenyl)piperazin-1-yl)ethyl]-4,4-dimethyl-1,3-(2H,4H)-isoquinolindione dihydrochloride) was purchased from Tocris Bioscience (Ellisville, MO, USA). Dexmedetomidine was purchased from Abbott Laboratories (Chicago, IL, USA). Pertussis toxin was purchased from List Biological Laboratories, Inc. (Campbell, CA, USA). All primary antibodies were bought from Abcam (Cambridge, MA, USA) and secondary antibodies were bought from Millipore (Billerica, MA, USA).

Stock solutions of dexmedetomidine was prepared in DMSO. A stock solution of indomethacin was prepared in 5 mM sodium bicarbonate solution. A stock solution of pertussis toxin was prepared in 0.1 M sodium sulfate buffer. All other solutions were prepared in deionized water. Concentrations are expressed as final molar concentration in the bath solution.

4.2. Porcine hearts

Pig hearts were collected from the local abattoir, where the animals were killed according to the regulations of the Food and Environmental Hygiene Department of the Hong Kong Special Administrative Region. The hearts were immediately rinsed and transported back to the laboratory in ice cold, oxygenated Krebs-Ringer solution (composition: NaCl, 118.3 mM; KCl, 4.7 mM; $MgSO_4$, 1.2 mM; KH_2PO_4, 1.2 mM; $CaCl_2$, 2.5 mM; $NaHCO_3$, 25.0 mM; glucose, 11.1 mM).

4.3. Large coronary arteries

The right coronary arteries were dissected free, separated from the surrounding fat and connective tissue, and cut into rings 4–5 mm in length. In some experiments, the endothelium was removed by perfusion with saponin in Krebs–Ringer solution (1 mg/mL, 1 mL) for 20 s before cutting the rings. The rings were suspended between stainless steel stirrups in jacketed organ chambers filled with Krebs-Ringer solution (5 mL), and the isometric contractile force was measured using a PowerLab 4SP data acquisition system (ADInstruments, Oxford, UK). The rings were subjected to a resting tension of 5 g, which was found to be the optimal tension in pilot experiments. The solution was maintained at 37 °C and continuously aerated with 95% O_2/5% CO_2.

4.4. Protocol for functional studies on porcine coronary arteries

After equilibration, indomethacin (10 mM) was added to the rings, with or without endothelium, the rings were contracted with prostaglandin F_{2a} (1–3 mM), and the relaxation to cumulative doses of dexmedetomidine (100 pM to 10 mM) was measured. Several antagonists were used to define the receptors and mechanisms involved. Rauwolscine (non-selective α_2-AR antagonist, 100 nM), BRL44408 (α_{2A}-AR antagonist, 3 nM – 300 nM), ARC239 (α_{2B}-AR antagonist, 10 nM – 1 mM) and MK912 (α_{2C}-AR antagonist, 0.03–3 nM), alone or in combination, were used to evaluate the role of different α_2-AR subtypes. N^w-nitro-L-arginine methyl ester (L-NAME, inhibitor of NO synthase, 100 mM) and pertussis toxin (inactivates G_i protein, 400 ng/mL) were used alone or in combination to evaluate the roles of NO and G_i-protein, respectively.

4.5. Characterization of a_2-adrenoreceptor subtype distribution

Studies on the anatomy of porcine coronary arteries have confirmed that coronary resistance arterioles run diffusely across the ventricular myocardium (Rodrigues et al. 2005). Tiny blocks of subendocardial myocardium, which contain numerous intramyocardial resistance coronary arterioles, were harvested from identical sites on the left ventricular wall. These blocks were then used to map out the distribution of different α_2-AR subtypes, using reverse transcription-polymerase chain reaction (RT-PCR), western blotting and immunofluorescence. The results were compared with those obtained using segments of porcine right coronary artery.

4.6. Reverse transcription-polymerase chain reaction

Total RNA was isolated from coronary arteries and subendocardial myocardium using TRIzol reagent (Invitrogen, Grand Island, NY, USA). Total RNA (2 mg) was used for first-strand cDNA synthesis, using random hexamer primers and Superscript II RNase H reverse transcriptase (SuperScript Preamplification System, Invitrogen). The resulting first-strand cDNA was directly used for PCR amplification.

Sets of primers were designed and synthesized for PCR analysis. The two primers used for amplifying α_{2A}-AR (accession no. NM_214400) were 5'-GAGCGCAG-GCCCAATGGCCTA-3' (sense) and 5'-GTTCTGCCTTCCGCGCCAGCG-3' (antisense), which generated a 363-bp PCR product. The two primers for α_{2B}-AR (accession no. DQ182110) were 5'-AGGGCTAAGGGGGGCCCTGGG-3' (sense) and 5'-GGTCAGCTGCGCCCGCCGACG-3' (antisense), which generated a 459-bp product. Reactions were carried out for 60 cycles with the following parameters: denaturation at 94 °C for 30 s, annealing at 50 °C for 1 min, and extension at 72 °C for 1.5 min. Porcine α_{2C}-AR has not been completed cloned so human primers were used instead. The two primers for α_{2C}-AR (accession no. NM_000683) were 5'-TGCGCGCGCCACAGAACCTCTTCCT-3' (sense) and 5'- ATGCAGGAGGA-CAGGATGTACCA-3' (antisense), which yielded a 403-bp PCR product (Mchrotra et al. 2006). Reactions were carried out for 60 cycles with the following parameters: denaturation at 94 °C for 45 s, annealing at 58 °C for 45 s, and extension at 72 °C for 1.5 min. PCR products were analyzed by agarose gel electrophoresis and visualized by staining with ethidium bromide.

4.7. Western blotting

Protein expression of α_2-ARs was determined by western blotting, as previously described (Li et al. 2019). Artery segments and blocks of myocardium were homogenized in ice-cold sodium phosphate buffer containing 1:1000 (v/v) protease inhibitor cocktail (5 mM, pH 8). The homogenate was centrifuged at 3000 × *g* for 10 min to remove nuclei and unbroken cells. The amount of protein in the supernatant was then determined using the Bradford protein assay. Proteins (20 mg) were resolved on 10% sodium dodecyl sulfate-polyacrylamide gels and electrotransferred to nitrocellulose membranes. The membranes were subsequently blocked with 5% (w/v) nonfat dry milk in PBS overnight at 4 °C, followed by incubation with polyclonal rabbit anti-α_{2A}-AR, anti-α_{2B}-AR or anti-α_{2C}-AR antibody [1:100 (v/v) dilution in blocking solution] at room temperature for 2 h. The nitrocellulose membranes were then washed extensively with 0.02% (v/v) Triton X-100 in PBS and incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody [1:5000 (v/v) dilution in blocking solution] at room temperature for 2 h. Excess secondary antibody was again removed by washing. The bound secondary antibody was detected by enhanced chemiluminescence. Protein expression of b-actin, which served as a positive control, was similarly detected using mouse monoclonal anti-b-actin antibody.

4.8. Immunofluorescence assay

Localization of different α_2-AR protein was investigated by immunofluorescence staining (Leung et al. 2001). Paraffin-embedded sections of paraformaldehyde-fixed coronary arteries and subendocardial myocardium (3 mm) were dewaxed and hydrated. Antigens were retrieved by treatment with citrate buffer (0.01 M, pH 6.0) for 5 min in a microwave oven. After rinsing with pure water and PBS, the sections were incubated in blocking serum for 30 min and then with rabbit polyclonal anti-α_{2A}-AR, anti-α_{2B}-AR or anti-α_{2C}-AR antibody diluted 1:100 (v/v) with diluting buffer (PBS with 0.01% (v/v) Triton X-100, 0.01% (v/v) Tween 20, and 0.1% (w/v) bovine serum albumin) at 4 °C overnight. Anti-von-Willebrand-factor-antibody was used as a marker for endothelial cells. Sections were washed three times with PBS and incubated with FITC- (for -α_{2A}-AR and anti-α_{2B}-AR) or rhodamine- (for α_{2C}-AR and von Willebrand factor) conjugated goat anti-rabbit secondary antibody [1: 100 (v/v) with diluting buffer]. The slides were then washed and mounted for observation with a confocal microscope.

4.9. Statistical analysis

Data are presented as the mean±S.E.M., with *n* representing the number of animals (pig hearts). For dose response curves, the concentration response curves were fitted to the Hill equation using Prism 4.0 (GraphPad Software, Inc., San Diego, CA, USA). In curves showing parallel rightward shifts without change in maximal response, antagonist affinities were estimated using the method of Arunlakshana and Schild (1959). If the Schild regression slope was not significantly different from unity, the antagonist affinity was estimated from the intercept of the regression line as described by Jenkinson and co-workers (1995).

Analysis of variance (ANOVA) was used for comparison of curves among multiple treatment groups. *Post hoc* comparison was performed by the Newman-Keuls multiple comparison test, where appropriate. Student's t-test was used in the case of two group comparisons. $P < 0.05$ was considered to indicate statistically significant differences.

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