Adenosine Receptor Regulation of Coronary Blood Flow in Ossabaw Miniature Swine^[S]

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ABSTRACT

Adenosine clearly regulates coronary blood flow (CBF); however, contributions of specific adenosine receptor (AR) subtypes (A_1 , A_{2A} , A_{2B} , A_3) to CBF in swine have not been determined. ARs generally decrease (A_1 , A_3) or increase (A_{2A} , A_{2B}) cyclic adenosine monophosphate, a major mediator of vasodilation. We hypothesized that A_1 antagonism potentiates coronary vasodilation and coronary stent deployment in dyslipidemic Ossabaw swine elicits impaired vasodilation to adenosine that is associated with increased A_1/A_{2A} expression. The left main coronary artery was accessed with a guiding catheter allowing intracoronary infusions. After placement of a flow wire into the left circumflex coronary artery the responses to bolus infusions of adenosine were obtained. Steady-state infusion of AR-specific agents was achieved by using a small catheter fed over the flow wire in control pigs. CBF was increased by the A_2 -nonselective agonist 2-phenylaminoadenosine (CV1808) in

a dose-dependent manner. Baseline CBF was increased by the highly A_1 -selective antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), but not changed by other AR-specific agents. The nonselective A_2 antagonist 3,7-dimethyl-1-propargylxanthine and A_{2A} -selective antagonist 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM241385) abolished adenosine-induced CBF, whereas A_{2B} and A_3 antagonism had no effect. Dyslipidemia and stenting decreased adenosine-induced CBF ~70%, whereas A_1 , A_{2A} , and A_{2B} mRNA were up-regulated in dyslipidemic versus control >5-fold and there was no change in the ratio of A_1/A_{2A} protein in microvessels distal to the stent. In control Ossabaw swine A_1 antagonism by DPCPX positively regulated basal CBF. Impaired adenosine-induced CBF after stenting in dyslipidemia is most likely caused by the altered balance between A_1 and A_{2A} signaling, not receptor expression.

Introduction

Adenosine contributes to the maintenance of coronary blood flow (CBF), especially during myocardial ischemia (Fredholm et al., 2001; Tune et al., 2004; Duncker and Bache,

2008) and is used clinically to elicit maximal vasodilation to diagnose flow-limiting conduit artery stenosis and microvascular dysfunction (Kern et al., 2006). Four different subtypes of adenosine receptors (ARs) have been cloned and pharmacologically characterized: A_1 , A_{2A} , A_{2B} , and A_3 (Fredholm et al., 2001; Shen et al., 2005). All are G protein-coupled and either inhibit (A_1 , A_3 , Gi/q) or stimulate (A_{2A} , A_{2B} , Gs) adenylyl cyclase (Fredholm et al., 2001). Adenosine predominantly dilates arterioles <100 μ m in diameter (Camici and Crea, 2007; Duncker and Bache, 2008). The vasodilatory mechanism involves A_2 activation and consequent elevation of cAMP-dependent protein kinase (PKA), resulting in vasodilation mainly via opening of ATP-sensitive K^+ (K_{ATP}) channels and voltage-dependent K^+ (K_v) channels (Standen and Quayle, 1998; Hein et al., 1999; Bender et al., 2009). Because A_1 decreases, A_2 increases cAMP (Fredholm et al., 2001) and the roles of A_1 and A_2 subtypes are opposing (Sato et al.,

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ABBREVIATIONS: CBF, coronary blood flow; A_1 , adenosine A_1 receptor; A_{2A} , adenosine A_{2A} receptor; A_{2B} , adenosine A_{2B} receptor; A_3 , adenosine A_3 receptor; AR, adenosine receptor; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; DMPX, 3,7-dimethyl-1-propargylxanthine; CCPA, 2-chloro-N(6)-cyclopentyladenosine; IVUS, intravascular ultrasound; APV, average peak velocity; HDL, high-density lipoprotein; LDL, low-density lipoprotein; PCR, polymerase chain reaction; ZM241385, 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol; MRS1706, N-(4-acetylphenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)phenoxy]acetamide; MRS1220, N-[9-chloro-2-(2-furyl)[1,2,4]triazolo[1,5-c]quinazolin-5-yl]benzene acetamide; CV1808, 2-phenylaminoadenosine.

2005), A_1 antagonism could positively modulate coronary vasodilation. Tawfik et al. (2006) provided compelling evidence for A_1 regulation of CBF in an A_1 knockout mouse, but, surprisingly, studies have not been done on A_1 in large animals that more closely mimic human coronary physiology. AR may be responsible for the dysregulated adenosine-induced CBF in dyslipidemia.

Deployment of a stent in a flow-limiting coronary atherosclerotic lesion of a conduit coronary artery results in substantial improvement in functional capacity and quality of life. However, almost one-third of patients undergoing coronary stenting eventually experience recurrent angina (Abbate et al., 2007). Beyond in-stent thrombosis/restenosis that is the most common cause of poststent angina (Abbate et al., 2007), downstream microvascular dysfunction has been implicated in causing exertional ischemia (van Liebergen et al., 1998; Kern et al., 1999; Monnick et al., 2003; Werner et al., 2004; Camici and Crea, 2007). This phenomenon was termed poststent "iatrogenic coronary microvascular dysfunction" (Camici and Crea, 2007). The mechanisms underlying microvascular dysfunction associated with stenting are unclear and deserve careful study. Patients with dyslipidemia exhibit impaired coronary flow reserve (Camici and Crea, 2007), which has also been shown convincingly in dyslipidemic swine models by in vivo flow measures (Zhu et al., 2009). Because AR functions are highly specific based on AR subtype, vascular bed, and species (Shen et al., 2005; Edwards et al., 2008), the vasodilatory effect of adenosine involves A_2 activation (Standen and Quayle, 1998; Hein et al., 1999; Bender et al., 2009), and A_1 and A_2 often mediate opposite effects of adenosine (Shen et al., 2005), the balance of A_1 and A_2 may contribute to impaired adenosine-induced CBF in chronic dyslipidemia after stenting.

We tested two central hypotheses in the present study: 1) A_1 antagonism potentiates coronary vasodilation in healthy control Ossabaw miniature swine, and 2) coronary stent deployment in dyslipidemic Ossabaw swine elicits impaired vasodilation to adenosine, which is associated with increased A_1/A_{2A} expression in the microvessels downstream from stented conduit coronary arteries.

Materials and Methods

Animal Care and Coronary Stenting. All protocols involving animals were approved by an Institutional Animal Care and Use Committee and complied fully with standards (National Research

Council, 1996; AVMA Panel on Euthanasia, 2001). Ten lean male Ossabaw miniature swine (age = 14 ± 4 months) were fed a diet consisting of standard chow with 22% kcal from protein, 70% kcal from carbohydrates, and 8% kcal from fat. The data in Figs. 1, 2, and 3 were obtained from those pigs. Another 10 male Ossabaw swine (age = 14 months; $n = 5$ /group) were randomized to lean control or dyslipidemic groups in the stenting study. The data in Figs. 4 and 5 were obtained from the pigs in the stenting study. Please see the details in the flow diagram of the study in Supplemental Fig. 1. The dyslipidemic pigs were fed chow supplemented with (percentage by weight): cholesterol 2.0, coconut oil 17, corn oil 2.5, and sodium cholate 0.7. This mixture yielded a diet of 13% kcal from protein, 40% kcal from carbohydrates, and 47% kcal from fat (Mokelke et al., 2005). Control and dyslipidemic groups were calorie-matched (3200 kcal/day) for 43 weeks until sacrifice, the intended experimental design to avoid development of obesity and metabolic syndrome (Sturek et al., 2007; Edwards et al., 2010). After 40 weeks on the diets, control and dyslipidemic pigs in the stenting study received a bare metal stent (2.5–4.0 mm in diameter by 8 mm in length; Express², Boston Scientific, Minneapolis, MN) in the circumflex coronary artery at a stent/artery ratio of 1.0 with optimal inflation pressure and recovered for 3 weeks before sacrifice as described previously (Sturek et al., 2007; Edwards et al., 2008, 2010; Lloyd et al., 2008; Long et al., 2010; Neeb et al., 2010). Swine received 325 mg of aspirin as antiplatelet therapy starting the day before the stenting and continuing for 3 weeks after stenting until completion of the study. There was no evidence of microvascular embolization in all animals. Cephalexin (1000 mg) was given twice a day for 6 days after the stent procedure. All pigs were housed and fed in individual pens under a 12-h light/12-h dark cycle. Water was provided ad libitum.

Coronary Blood Flow. The pigs were preanesthetized by using telazol/xylazine and maintained by using 2 to 4% isoflurane with oxygen via tracheal intubation and ventilation at a tidal volume of typically ~700 ml (10–15 ml/kg) and respiratory rate of 15/min. Heart rate, blood pressure, oxygen saturation, and electrocardiographic data were continuously monitored. These precautions confirmed normal oxygen saturation and no hypercarbia or acidosis among the pigs based on blood gas and pH analysis. The right femoral artery was accessed by surgical cut-down and an 8 F vascular introducer sheath was inserted followed by administration of heparin (200 U/kg). A Cordis (Bridgewater, NJ) 8 F Amplatz L (sizes 0.75–2.0) guiding catheter was advanced into the aortic arch, and a guiding catheter was engaged with the ostium of the left main artery. A 0.014-inch diameter Doppler flow wire (JoMed Inc., Rancho Cordova, CA) or combination pressure and flow wire (ComboMap; Volcano Therapeutics, Inc., Rancho Cordova, CA) was advanced down the circumflex artery under angiographic guidance and a nonbranching section was selected for baseline and drug-induced flow measurements (Mokelke et al., 2005; Sturek et al., 2007; Long et al., 2010; Neeb et al., 2010). Anatomical landmarks were noted in case the wire

Fig. 1. Vasodilatory effect of adenosine mediated by A_2 . A and B, effects of continuous intracoronary infusion of A_2 -nonselective antagonist DMPX on base APV (A) and peak APV (B) during concurrent bolus injection of 0, 0.167, 0.33, or 1.0 $\mu\text{g/kg}$ adenosine ($n = 5$). "Ado only" in B denotes the base APV between bolus doses of Ado. Flow signal was measured with an intracoronary flow wire placed under the guidance of coronary angiogram as described under *Materials and Methods*. C, effects of intracoronary bolus injection of the A_2 -nonselective agonist CV1808 on percentage of APV increase (percentage APV increase = [peak APV – base APV]/base APV $\times$ 100; $n = 5$). Base indicates the APV readings before adenosine administration. Ado only means pure adenosine dose response, and Ado+DMPX means adenosine dose response with concurrent continuous infusion of DMPX. *, $p < 0.05$; ***, $p < 0.001$, versus Ado only at corresponding dose or as indicated by lines.

moved and required repositioning. In all lean control/stent (see Fig. 4, $n = 5$), all dyslipidemia/stent (see Fig. 4, $n = 5$), lean/nonstent during A_{2A} -, A_{2B} -, and A_3 -selective antagonist administration (Fig. 2, $n = 5$) an intravascular ultrasound (IVUS; 3.2 F 30 MHz; Boston Scientific, Inc.) catheter was then advanced over the flow wire and positioned in the circumflex to align the IVUS imaging transducer with the flow wire tip to obtain accurate diameter measurement of the artery at the location where the flow velocity measurements were acquired (Mokelke et al., 2005). In the experiments in which IVUS was not used to determine conduit artery area, angiography was used to confirm that conduit artery diameter did not change in response to adenosine receptor modulation; nonetheless, we conservatively expressed the coronary blood flow changes as velocity, not volume flow. For constant infusion of drugs the IVUS catheter was placed with only the tip in the most proximal circumflex and drugs were infused through the IVUS catheter. Flow velocity signals were allowed to stabilize for several minutes for determination of CBF velocity responses. For the pigs with stent deployment, coronary blood flow analysis of in vivo microvascular function was done 3 weeks after stenting and in a nonbranching section of the circumflex artery proximal to the stent.

Hyperemia was induced with 3-ml bolus doses of the endothelium-independent vasodilator adenosine (0.167, 0.33, and 1 $\mu\text{g}/\text{kg}$) given via the guiding catheter into the coronary artery as described previously (Mokelke et al., 2005) and is identical to clinical catheterization assessment in humans (Kern et al., 2006). Adenosine injection was followed by 10 ml of saline flush. Saline artifact was tested in each animal and, if present (usually less than 10% peak flow induced by adenosine), was subtracted from subsequent flow measurements. Peak average peak velocity (APV), heart rate, and blood pressure were recorded for each adenosine administration. Each APV value was calculated on-line by using the automated data acquisition system as an average of instantaneous CBF velocity over two consecutive cardiac cycles. Subsequent doses of adenosine were administered when APV, heart rate, and blood pressure had returned to baseline and stabilized, at which time the baseline parameters were again documented followed by the administration of the next adenosine dose. The effect of adenosine typically lasted 15 to 30 s.

The adenosine A_2 -nonselective agonist 2-phenylaminoadenosine (CV1808) (10^{-6} , 10^{-5} , and 10^{-4} M) was administered via bolus injection as was done with adenosine. The IVUS catheter was placed in the proximal circumflex coronary artery in control swine for steady-state infusion of selected drugs at a rate of 1 ml/min to observe their effects over a relatively longer period of time: 2-chloro- N (6)-cyclopentyladenosine (CCPA), 10^{-5} M, A_1 -selective agonist, Tocris Bioscience, Ellisville, MO; 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), 10^{-4} M, A_1 -selective antagonist, Tocris Bioscience; 3,7-dimethyl-1-propargylxanthine (DMPX), 10^{-4} M, A_2 -nonselective antagonist, Sigma-Aldrich, St. Louis, MO; 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM241385),

10^{-7} M, A_{2A} -selective antagonist, Tocris Bioscience; N -(4-acetylphenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1*H*-purin-8-yl)phenoxy]acetamide (MRS1706), 10^{-7} M, A_{2B} -selective antagonist, Tocris Bioscience; N -[9-chloro-2-(2-furyl)[1,2,4]-triazolo[1,5-*c*]quinazolin-5-yl]benzene acetamide (MRS1220), 10^{-7} M, A_3 -selective antagonist, Tocris Bioscience. This was followed by concurrent bolus injection of adenosine. Vehicle control (1% dimethyl sulfoxide) for DMPX was not used in parallel, which should be used for future experiments. The vehicle controls for other reagents were either water or phosphate-buffered saline. Continuous saline infusion was done between each constant infusion treatment to make sure the flow returned to baseline. The above-mentioned drug infusions were done in random order in each pig.

The analog Doppler signals were continuously digitized both as instantaneous CBF velocity and APV values. All flow data were stored on videotapes and personal computers for additional off-line analysis. Data are also expressed as percentage of APV increase, which equals $[\text{peak APV} - \text{base APV}]/\text{base APV} \times 100$. Volumetric CBF was calculated as: $\text{CBF (in ml/min)} = (\text{artery cross-sectional area in cm}^2) \times (\text{velocity in cm/s}) \times 0.5 \times 60 \text{ s/min}$ (Mokelke et al., 2005). Vessel cross-sectional area was calculated off-line from recorded IVUS images by using the commercially available Sonos Intravascular Imaging software package (Hewlett Packard, Palo Alto, CA). Angiography and IVUS showed no change in conduit artery diameter during any drug exposures, thus coronary flow velocity represented microvascular effects.

Plasma Lipid Assays. Venous blood samples were obtained after overnight fasting and were analyzed for triglyceride and total cholesterol [fractionated into high-density lipoprotein (HDL) and low-density lipoprotein (LDL) components] (Edwards et al., 2010).

Real-Time Reverse Transcription-Polymerase Chain Reaction. RNA was extracted from coronary microvessels downstream of left circumflex coronary artery by using TRIzol (Invitrogen, Carlsbad, CA), then treated with DNase (DNA free; Ambion, Austin, TX) to remove contaminating genomic DNA, and analyzed by NanoDrop spectrophotometer (ND-1000; Thermo Fisher Scientific, Wilmington, DE) to assess purity (A260/280) and concentration. RNA (1 μg) was converted to cDNA by using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) in a standard thermocycler (DNA Engine PTC-200; MJ Research, Watertown, MA). A_1 , A_{2A} , A_{2B} , and A_3 mRNA expression was assessed by using quantitative real-time reverse transcription-PCR in an ABI 7500 instrument (Applied Biosystems, Foster City, CA). Primers for PCR amplification were as follows: A_1 , forward, 5'-GGCCATGCTGGCAATTG-3', reverse, 5'-CCT-GAGCGGATCTTGACA-3'; A_{2A} , forward, 5'-CCCCTTCATCTATGCTACCG-3', reverse, 5'-CATTCCCTCACACTCCCTCCAC-3'; A_{2B} , forward, 5'-TGTGTCTGGTGCCTCTTCAC-3', reverse, 5'-ACACGATGGGGTTGACGACC-3'; A_3 , forward, 5'-GAACCTCACCTTCCTTTCCTGC-3', reverse, 5'-GAACTCCCGTCCATAAAATGC-3' (Integrated DNA Technologies, Coralville, IA). Taqman probes were used to

Fig. 2. A_{2A} , not A_3 , contributed to adenosine-induced vasodilation. Base and peak coronary blood flow measurement in typical adenosine dose responses was done in lean animals ($n = 5$) as described in Fig. 1, except that conduit diameter measures enabled calculation of volumetric blood flow. Effects of continuous intracoronary infusion of vehicle control, A_{2A} -selective antagonist ZM241385, and A_{2B} -selective antagonist MRS1706 (A) or A_3 -selective antagonist MRS1220 (B) on peak coronary blood flow during concurrent bolus injection of adenosine at 0.167, 0.33, or 1.0 $\mu\text{g}/\text{kg}$ are depicted. Peak CBF was compared between A_{2A}/A_{2B} antagonism (A) or A_3 antagonism (B) and vehicle control at the same adenosine doses. *, $p < 0.05$; #, $p < 0.001$, ZM241385 versus vehicle control (A) at corresponding adenosine doses.

detect A_1 gene amplification and were as follows: 5'-/6-FAM/TCGACCGCTACCTCC/MGBNFQ/-3' (Applied Biosystems). SYBR Green Universal PCR Master Mix (Applied Biosystems) was used to detect A_{2A} , A_{2B} , and A_3 gene expression followed by a dissociation curve to rule out nonspecific amplifications and primer dimers. All results were normalized to the signal of 18s RNA, which was amplified in separate reactions by using TaqMan Universal PCR Master Mix and TaqMan 18s Detection Reagents (Applied Biosystems).

Immunohistochemistry for AR. Left ventricular myocardium was preserved in 10% formalin solution, embedded in paraffin, sectioned, and treated with antibodies similar to our previous methods (Turk et al., 2003). Primary polyclonal antibodies to A_1 and A_{2A} were applied and incubated overnight (1:100 dilution; Novus Biologicals Inc., Littleton, CO). A biotinylated secondary antibody (Vectastain Elite; Vector Laboratories, Burlingame, CA) was used for the detection process. Quantification of AR staining in each microvessel was accomplished with commercially available software (Image Pro Plus, 4.1; MediaCybernetics Inc., Bethesda, MD).

Data Analysis. Data are presented as means $\pm$ S.E.M. Statistical analysis was performed with commercially available software [(SPSS version 12 (SPSS Inc., Chicago, IL) and Prism version 4.0 (GraphPad Software Inc., San Diego, CA)]. Means of more than two groups were compared by one-way analysis of variance with least significant difference post hoc analyses (by SPSS) or two-way analysis of variance followed by Bonferroni post-test (by Prism). Means of two groups were compared by Student's *t* test (unpaired, two-tailed). In all cases, $p < 0.05$ was considered statistically significant.

Results

Metabolic Characteristics of Control and Dyslipidemic Pigs with Coronary Stent Deployment. The atherogenic diet resulted in significantly higher plasma total cholesterol, LDL, HDL, and LDL/HDL versus control pigs fed standard chow diet (Table 1). There were no differences in body weight, plasma triglycerides, fasting plasma insulin, fasting plasma glucose (Table 1), or resting blood pressure (Table 2). The plasma lipid profile defined the atherogenic diet-fed group as dyslipidemic, but not having metabolic syndrome.

Contribution of A_2 to Coronary Blood Flow Regulation. The A_2 -nonselective antagonist DMPX did not change base APV response (Fig. 1A), but abolished peak APV response (Fig. 1B) to adenosine at 0.33 and 1.0 $\mu\text{g/kg}$. The A_2 -nonselective agonist CV1808 elevated the percentage of APV increase ($[\text{peak APV} - \text{base APV}] / \text{base APV} \times 100$) in a dose-dependent manner in the absence of exogenous adenosine (Fig. 1C). The CV1808-induced percentage of APV increase at 10^{-4} M was comparable with adenosine-induced percentage of APV increase at 1.0 $\mu\text{g/kg}$, which was calculated from the base and peak APV in Fig. 1, A and B separately.

TABLE 1

Metabolic data from control and dyslipidemic pigs with stent deployment

Metabolic Panel	Control Pigs	Dyslipidemic Pigs
Body weight (kg)	74 $\pm$ 2	76 $\pm$ 2
Fasting plasma glucose ($\mu\text{U/ml}$)	74 $\pm$ 4	86 $\pm$ 5
Fasting plasma insulin (mg/dl)	7 $\pm$ 1	10 $\pm$ 2
Total cholesterol (mg/dl)	62 $\pm$ 3	299 $\pm$ 33*
LDL (mg/dl)	24 $\pm$ 2	216 $\pm$ 34*
HDL (mg/dl)	34 $\pm$ 1	73 $\pm$ 12*
Triglycerides (mg/dl)	19 $\pm$ 3	45 $\pm$ 12
LDL/HDL	0.7 $\pm$ 0.1	3.6 $\pm$ 1.1*

* significantly different ($P < 0.05$) vs. control.

TABLE 2

Hemodynamic characteristics comparison during coronary blood flow measures in control and dyslipidemic pigs 3 weeks after stenting

Hemodynamics	Control Pigs	Dyslipidemic Pigs
HR (bpm)	101 $\pm$ 4	109 $\pm$ 4
SBP (mm Hg)	75 $\pm$ 3	76 $\pm$ 3
DBP (mm Hg)	53 $\pm$ 3	53 $\pm$ 3
MAP (mm Hg)	60 $\pm$ 3	61 $\pm$ 3
RPP (bpm*mm Hg)	7610 $\pm$ 820	8340 $\pm$ 820
CBF (ml/min)	46 $\pm$ 7	42 $\pm$ 6
CSA (mm ²)	10 $\pm$ 1	9 $\pm$ 1

HR, heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; RPP, rate pressure product; CSA, vessel cross-sectional area.

Contribution of A_{2A} , A_{2B} , and A_3 to Coronary Blood Flow Regulation. Figure 2 displays data as absolute blood flow in pigs with conduit diameter accurately measured by using IVUS before flow measures. Bolus injection of adenosine in the presence of vehicle control increased peak APV versus base in a dose-dependent manner (Table 3). Continuous intracoronary infusion of the A_{2A} -selective antagonist ZM241385 abolished the adenosine-induced peak CBF (Fig. 2A), while having no effect on baseline CBF (data not shown). The A_{2B} -selective antagonist MRS1706 (Fig. 2A) and A_3 -selective antagonist MRS1220 (Fig. 2B) had modest effects on the adenosine-induced peak CBF compared with ZM241385 and no effect on base CBF (data not shown).

Contribution of A_1 to Coronary Blood Flow. Constant infusion of the highly A_1 -selective antagonist DPCPX increased base APV without exogenous adenosine and increased peak APV response to adenosine at 1.0 $\mu\text{g/kg}$ (Fig. 3). Intracoronary infusion of the highly A_1 -selective agonist CCPA did not alter base APV in the absence of exogenous adenosine or peak APV response to adenosine (Fig. 3). There was no change of systemic parameters (i.e., blood pressure, heart rate) by the administration of AR agonists/antagonists in the nonstented lean control pigs (data not shown; Mokelke et al., 2005; Long et al., 2010).

Comparison of Coronary Blood Flow in Control and Dyslipidemic Pigs Undergoing Stenting. There were no significant differences in hemodynamic characteristics across groups (Table 2), e.g., heart rate, mean arterial pressure, rate pressure product, and baseline CBF. The CBF (Fig. 4) response to adenosine infusion at 0.167 and 1.0 $\mu\text{g/kg}$ was blunted in dyslipidemic versus control pigs 3 weeks after stenting.

Expression of AR in Coronary Microvessels of Control and Dyslipidemic Pigs 3 Weeks after the Stent Deployment. A_1 , A_{2A} , and A_{2B} mRNA expression was up-regulated >5 -fold in coronary microvessels downstream from the stented left circumflex coronary artery in dyslipidemic pigs compared with control pigs (Fig. 5, A–C). A_3 mRNA level

TABLE 3

Typical adenosine dose responses

Adenosine Dose	Base APV	Peak APV
$\mu\text{g/kg}$	cm/s	
0.167	2.4 $\pm$ 2.0	23.3 $\pm$ 4.7*
0.33	13.0 $\pm$ 1.8	27.0 $\pm$ 4.2*
1.0	12.8 $\pm$ 0.9	34.0 $\pm$ 2.0*

* indicates $P < 0.05$ and # indicates $P < 0.001$, both significantly different vs. base APV, where base means the APV readings before adenosine administration.

was not different between groups (Fig. 5D). Immunohistochemistry showed that although there was a trend for an increase in A_1/A_{2A} protein expression in microvessels of hyperlipidemic pigs (1.12 ± 0.04) versus lean control pigs (1.0 ± 0.05), the difference was not significant ($P > 0.05$).

Fig. 3. Contribution of A_1 to coronary blood flow regulation. Highly A_1 -selective agonist CCPA, A_1 -selective antagonist DPCPX, or vehicle control was constantly infused through an intracoronary catheter. After coronary blood flow stabilized, base APV was measured ($n = 10$). Concurrent bolus injection of adenosine at 0.167, 0.33, or 1.0 $\mu\text{g/kg}$ was done afterward, and flow signal was measured as in Fig. 1. Effects of CCPA and DPCPX on peak APV on adenosine bolus injection are depicted versus vehicle control. *, $p < 0.05$ versus vehicle control at the corresponding adenosine dose.

Fig. 4. Adenosine-induced coronary flow in control and dyslipidemic pigs 3 weeks after stent deployment. Flow signal was measured in a non-branching section of the left circumflex coronary artery in lean control and dyslipidemic pigs 3 weeks after stenting ($n = 5/\text{group}$). Bolus injection of adenosine at 0.167, 0.33, or 1.0 $\mu\text{g/kg}$ was done as in Fig. 2 to elicit peak CBF response. Change of CBF = peak CBF – base CBF. *, $p < 0.05$ versus control.

Fig. 5. AR mRNA expression in coronary microvessels in control and dyslipidemic pigs 3 weeks after stent deployment. Expression levels of A_1 , A_{2A} , A_{2B} , and A_3 mRNA in A, B, C, and D, respectively, were determined by real-time reverse transcription-PCR in total RNA isolated from coronary microvessels distal from stented coronary conduit segments in lean control and dyslipidemic pigs ($n = 5/\text{group}$). *, $p < 0.05$ versus control.

Discussion

We present several key findings on adenosine in regulation of CBF. First, adenosine-induced vasodilation was mediated almost exclusively by A_{2A} in Ossabaw swine. Second, A_1 antagonism augments vasodilatory effects of some vasodilators other than adenosine in porcine coronary microvessels under basal conditions in vivo. Third, bare metal stent deployment in coronary conduit arteries in dyslipidemic Ossabaw swine elicited microvascular dysfunction, which was associated with increased A_1 and, paradoxically, increased A_{2A} and A_{2B} mRNA expression. Collectively, these data emphasize the delicate balance between A_1 and A_{2A} signaling in control of CBF in the setting of dyslipidemia and stenting.

Adenosine was shown as a vasodilator in porcine coronary microcirculation via activation of A_{2A} (Hein and Kuo, 1999). The A_{2B} subtype was suggested to mediate vasodilation of adenosine in human small coronary arteries (Kemp and Cocks, 1999), rat coronary circulation (Hinschen et al., 2003), and murine heart (Talukder et al., 2003). A_3 either inhibited or negatively modulated the vasodilatory effect of adenosine in mouse coronary circulation (Talukder et al., 2002). Studies in pigs (Wang et al., 2005) and humans (Sato et al., 2005) demonstrated A_1 mRNA and protein expression in coronary arterioles. In our current study, the nonselective A_2 antagonist DMPX did not change base APV (Fig. 1A); therefore A_2 were not tonically activated under normal, basal conditions. The A_{2A} -selective antagonist ZM241385 decreased peak CBF induced by exogenous adenosine (Fig. 2A), whereas the A_{2B} -selective antagonist MRS1706 and A_3 -selective antagonist MRS1220 showed relatively little effect on CBF (Fig. 2), thus providing evidence that mainly A_{2A} mediates vasodilatory effects of exogenous adenosine in coronary microcirculation of Ossabaw swine.

The highly A_1 -selective antagonist DPCPX increased base APV without exogenous adenosine (Fig. 3), whereas A_2 were not tonically activated, indicating A_1 antagonism by DPCPX might potentiate vasodilatory effects of some physiologically active vasodilators other than adenosine. In contrast, the highly A_1 -selective agonist CCPA did not change APV with or without exogenous adenosine. There are several explanations for the phenomena. First, CCPA effects could be

masked by endogenous adenosine. Because the binding affinity of adenosine for A_1 is higher than for A_2 , and the desensitization of A_1 is much slower than A_2 ($t_{1/2} = 10$ h versus 20 min) (Stiles, 1992), it could be that tonically A_1 was activated by adenosine, whereas A_2 was not. Therefore, endogenous adenosine activation of A_1 could shadow exogenous CCPA activation of A_1 . Second, we used 10^{-5} M CCPA for steady-state intracoronary infusion (1 ml/min), which was diluted by continuous coronary blood flow (~ 60 ml/min). Therefore the actual intracoronary concentration of CCPA might not be effective enough to regulate CBF. Research done in isolated hearts from A_1 knockout and wild-type mice showed that lower concentrations (10^{-9} to 10^{-7} M) of CCPA did not significantly change CBF (Tawfik et al., 2006). Intracoronary infusion of CCPA at different concentrations may help to address that in the future. Third, because A_2 were not tonically activated, adenosine might not play an important role in the maintenance of CBF under basal conditions, which was echoed by numerous studies done in normal hearts of dogs, swine, or humans under basal conditions or exercise hyperemia (Tune et al., 2002, 2004; Duncker and Bache, 2008). However, adenosine played an important role in coronary vasodilation when the myocardium was ischemic or hypoxic, which is accompanied by abundant adenosine release (Tune et al., 2002, 2004; Sato et al., 2005; Duncker and Bache, 2008).

Compared with other animal models Ossabaw swine are predisposed to metabolic syndrome (Sturek et al., 2007; Neeb et al., 2010) and have substantial diffuse and focal, flow-limiting coronary atherosclerosis (Edwards et al., 2010); therefore, stenting in the Ossabaw swine is quite clinically relevant. Dyslipidemia in the present study was manifested as described previously (Sturek et al., 2007; Bender et al., 2009; Edwards et al., 2010). The reason metabolic syndrome or obesity was avoided in the animals was to reduce the factors that may complicate the results, thus allowing the focus on dyslipidemia. It is noteworthy that A_1 , A_{2A} , and A_{2B} mRNAs all were up-regulated in coronary microvessels distal to the stent in dyslipidemic swine versus control (Fig. 5). Why was the adenosine-induced CBF response attenuated with A_{2A} and A_{2B} mRNA up-regulation in dyslipidemic swine? There are several explanations. First, mRNA does not always correspond directly to protein, as shown by the decrease in porcine coronary arteriolar A_{2B} protein expression in metabolic syndrome (Bender et al., 2009). Second, A_{2A} and A_{2B} proteins may not function normally. The similar coronary microvascular A_1/A_{2A} protein expression determined by immunohistochemistry in control versus dyslipidemic pigs suggests an altered balance between A_1 and A_{2A} signaling. The muted response to adenosine challenge is much more likely to be the result of signaling such as altered receptor coupling (e.g., via G proteins), cAMP formation, or other downstream messengers. Future studies should address downstream signal transduction pathways. Third, possible elevation of adenosine in dyslipidemia, stenting, and ischemia could result in high-affinity binding and activation of the A_1 receptor that overwhelms the vasodilatory effect mediated by A_{2A} activation. This is feasible because the affinity of adenosine for A_1 is higher than A_2 and the desensitization of A_1 is much slower than A_2 ($t_{1/2} = 10$ h versus 20 min) (Stiles, 1992). The net result is greater coupling of adenosine to A_1 than to A_2 to regulate CBF. Fourth, analogous to other disease states,

vasodilator signaling pathways can be up-regulated to partially compensate for increased vasoconstrictor or decreases in other vasodilator signaling pathways. For example, Ca^{2+} -dependent K^+ channel function is up-regulated in hypertension, but does not fully compensate for vasoconstrictor influences (Liu et al., 1995).

Adenosine has been found to increase postischemic recovery of function in humans in clinical interventions, such as angioplasty (Mubagwa and Flameng, 2001). These effects may be largely caused by cardiac muscle A_1 . Furthermore, the modulation/attenuation of heart rate (especially β -adrenergic stimulated) is also a classic finding (Belardinelli et al., 1989). There was no change of systemic parameters (i.e., blood pressure, heart rate) by the administration of AR agonists/antagonists (Mokelke et al., 2005; Long et al., 2010); therefore, under the conditions used for our flow measures (anesthetized, near resting heart rate and blood pressure) there was no direct effect of adenosine on cardiac muscle to change cardiac metabolism and, in turn, coronary blood flow. These data do not entirely rule out a direct inhibitory effect of adenosine on cardiac myocytes at higher heart rates elicited by β -adrenergic stimulation and the possibility of different adenosine reduction of heart rate in lean control versus dyslipidemic groups. Future studies should involve adrenergic provocation challenge with adenosine administration in the dyslipidemic pigs.

To our knowledge, this is one of the few studies examining the effects of stenting on coronary microcirculation in a porcine model of dyslipidemia (Long et al., 2010). Because isoflurane was used in each group of animals during the procedures, its activation of coronary K_{ATP} channels will elicit more baseline coronary vasodilation perhaps compared with other anesthetics (Kersten et al., 1997); however, the differences between groups should be caused more by the dyslipidemia and stent treatment than isoflurane administration. Stenting and dyslipidemia are two possible causes for the dysregulated adenosine-induced CBF in dyslipidemic Ossabaw swine 3 weeks after stent deployment (Fig. 4). Despite the findings that adenosine receptor-mediated coronary flow was impaired in dyslipidemic patients and patients with metabolic syndrome (Camici and Crea, 2007), as well as in dyslipidemic swine models (Zhu et al., 2009), previous study in our laboratory showed that diabetic dyslipidemic Yucatan pigs (Mokelke et al., 2005) and dyslipidemic Ossabaw pigs (Bender et al., 2009; Neeb et al., 2010) did not exhibit impaired microvascular dilation to adenosine. Coronary stenting has been shown to mechanically damage vascular cells in the target conduit artery segment and endothelium in peristent segments (Kim et al., 2009; Long et al., 2010) and induce downstream microvascular dysfunction (van Liebergen et al., 1998; Kern et al., 1999; Monnink et al., 2003; Werner et al., 2004; Camici and Crea, 2007). However, none of those stent studies were done in a swine model of dyslipidemia prone to metabolic syndrome and the microcirculatory dysfunction was either attributable to endothelium (Monnink et al., 2003) or unclarified (van Liebergen et al., 1998; Kern et al., 1999; Werner et al., 2004). Further studies are needed to clarify 1) whether dyslipidemia or stenting alone or the combination is the cause for the impaired adenosine-induced flow in the setting of dyslipidemia and stenting, 2) which adenosine receptor downstream signaling events are responsible for the adenosine-induced coronary flow dysregulation in dyslipide-

mia and stenting, and 3) whether the classic adenosine attenuation of β -adrenergic receptor-induced increases in heart rate affects coronary blood flow differently in control versus dyslipidemic pigs.

We conclude that A_1 antagonism by DPCPX promotes vasodilation in "healthy" anesthetized Ossabaw swine. An imbalance of A_1/A_{2A} signaling in coronary microcirculation distal to a stent was associated with dysregulated adenosine-induced CBF in dyslipidemia. The use of novel adenosine analogs for vasodilation in disease deserves further pharmacological and mechanistic study.

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