J Comp Physiol B (1999) 169: 237–248

ORIGINAL PAPER

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Cardiovascular control via angiotensin II and circulating catecholamines in the spiny dogfish, *Squalus acanthias*

Accepted: 24 February 1999

Abstract The contributions of circulating angiotensin II (Ang II) and catecholamines to cardiovascular control in the spiny dogfish were investigated by monitoring the effects of exogenous and endogenous dogfish [Asn^1, Pro^3, Ile^5]-Ang II (dfAng II) on plasma catecholamine levels and blood pressure regulation. Bolus intravenous injections of dfAng II (30–1200 pmol kg^{-1}) elicited dose-dependent increases in plasma adrenaline and noradrenaline concentrations, caudal artery pressure (P_{CA}), and systemic vascular resistance (R_S), and a decrease in cardiac output (Q). Similar injections of Ang II in dogfish pre-treated with the α-adrenoceptor antagonist yohimbine (4 mg kg^{-1}) also elicited dose-dependent increases in plasma catecholamine levels yet the cardiovascular effects were abolished. Dogfish treated with yohimbine were hypotensive and had elevated levels of plasma Ang II and catecholamines. Intravenous injection of the smooth muscle relaxant papaverine (10 mg kg^{-1}) elicited a transient decrease in P_{CA} and R_S, and increases in plasma Ang II and catecholamine levels. In dogfish first treated with lisinopril (10^{-4} mol kg^{-1}), an angiotensin converting enzyme inhibitor, papaverine treatment caused a more prolonged and greater decrease in P_{CA} and R_S, an attenuated increase in plasma catecholamines, and no change in plasma Ang II. By itself, lisinopril treatment had little effect on P_{CA}, and no effect on R_S, plasma Ang II or catecholamines. In yohimbine-treated dogfish, papaverine treatment elicited marked decreases in P_{CA}, R_S, and Q, and increases in plasma Ang II and catecholamines. Among the three papaverine treatments, there was a positive linear relationship between plasma Ang II and catecholamine concentrations, and the cardiovascular and hormonal changes were most pronounced in the yohimbine + papaverine treatment. Therefore, under resting normotensive conditions, while Ang II does not appear to be involved in cardiovascular control, catecholamines play an important role. However, during a hypotensive stress elicited by vascular smooth muscle relaxation, Ang II indirectly contributes to cardiovascular control by dose-dependently stimulating catecholamine release.

Key words Catecholamines · Angiotensin II · Cardiovascular control · Hypotension · Spiny dogfish, *Squalus acanthias*

Abbreviations *ACE* angiotensin converting enzyme · *Ang I* angiotensin I · *Ang II* angiotensin II · *dfAng II* dogfish angiotensin II · *EDTA* ethylenediaminetetraacetic acid · f_H heart rate P_{CA} mean caudal artery pressure · Q cardiac output *RAS* renin-angiotensin system · R_S systemic vascular resistance · V_S stroke volume

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Introduction

Throughout the vertebrates, the adrenergic and renin-angiotensin systems (RAS) play important roles in cardiovascular homeostasis. However, comparative studies have revealed that the mode of action of either the RAS or the adrenergic system, and the degree of interaction between the two, varies considerably within the vertebrate lineage (Morris and Nilsson 1994; Kobayashi and

Takei 1996). In elasmobranchs, some of the major visceral arteries receive adrenergic innervation (Nilsson et al. 1975), but adrenergic nerves are absent from the heart (Short et al. 1977) and do not appear to play a significant role in the control of the systemic circulation (Opdyke et al. 1972; Holcombe et al. 1980). Catecholamine injections, in contrast, elicit systemic vasoconstriction in elasmobranchs (Capra and Satchell 1977a; Kent and Pierce 1978; Opdyke et al. 1982), and there is pharmacological evidence suggesting that circulating catecholamines may be important for maintaining basal cardiac activity and systemic vascular resistance (Opdyke et al. 1972; Short et al. 1977). Hence, although direct evidence is scarce, the adrenergic contribution to cardiovascular control in elasmobranchs appears to be primarily humoral (Butler and Metcalfe 1988), unlike the situation in most vertebrates (Morris and Nilsson 1994).

While previously debated (see Olson 1992, Henderson et al. 1993 and Kobayashi and Takei 1996 for reviews), the presence of an RAS in elasmobranchs was recently confirmed by the isolation of angiotensin I (Ang I) from the dogfish, *Triakis scyllia* (Takei et al. 1993). Evidence for an involvement of the RAS in the cardiovascular control of elasmobranchs is both direct and indirect. As in other vertebrates, angiotensin II (Ang II), the active product of the RAS, has vasopressor activity in dogfish (Opdyke and Holcombe 1976; Khosla et al. 1983; Hazon et al. 1995; Tierney et al. 1997). In teleosts and elasmobranchs, as in mammals (Butler et al. 1994), the results of numerous studies indicate that a varying portion of Ang II-mediated vasopressor activity is linked to an interaction with the adrenergic system (Opdyke and Holcombe 1976; Carroll 1981; Opdyke et al. 1981, 1982; Carroll and Opdyke 1982; Khosla et al. 1983; Bernier and Perry 1999). Only a single study (Tierney et al. 1997) has attributed the vasopressor effects of Ang II exclusively to its direct actions on the cardiovasculature.

Moreover, there is evidence that Ang II may play a role in cardiovascular control in elasmobranchs during a hypotensive stress (Hazon et al. 1989; Galli and Kiang 1990). However, the direct involvement and mode of action of either circulating Ang II or catecholamines under such conditions have yet to be assessed. Specifically, it has not yet been established whether Ang II *directly* contributes to cardiovascular control during hypotension or has an *indirect* role via its activation of the humoral adrenergic system. The primary objective of this study, therefore, was to assess the relative contributions of circulating Ang II and catecholamines to cardiovascular control during hypotensive conditions in the dogfish *Squalus acanthias*.

Materials and methods

Experimental animals

Spiny dogfish, *Squalus acanthias*, of either sex weighing between 1130 g and 2355 g (experimental $n = 30$) were collected by net during trawl by local fisherman and transported to holding facilities at Bamfield Marine Station (Bamfield, British Columbia). The dogfish were kept under natural photoperiod in a 75,000 l opaque circular tank provided with aerated full-strength sea water at 11 °C. They were fed twice weekly with herring and used for experimentation within 4 weeks of capture.

Surgical procedures

Dogfish were immersed in an aerated anaesthetic solution of ethyl-*m*-aminobenzoate (0.1 g l^{-1}; MS-222; Syndel, Vancouver, BC) and transferred to an operating table where the gills were irrigated continuously with the same anaesthetic solution. A lateral incision was made in the caudal peduncle to expose and cannulate (PE 50; Clay Adams) both the caudal vein and the caudal artery in the anterograde direction. While the arterial cannula allowed caudal artery blood pressure (P_{CA}) measurements, the caudal vein cannula permitted injections and repeated blood sampling. Both cannulae were filled with heparinised (100 IU ml^{-1} sodium heparin; Sigma Chemical, St. Louis, Mo.) dogfish saline (500 mmol l^{-1} NaCl). In addition, the pericardial cavity was exposed with a ventral midline incision and the pericardium was dissected to expose the conus arteriosus. To enable measurement of cardiac output (Q), a 3S or 4S ultrasonic flow probe (Transonic Systems, Ithaca, N.Y.) was placed loosely and non-occlusively around the conus. Lubricating jelly was used with the perivascular flowprobe as an acoustic coupler. Silk sutures were used to close the ventral and caudal peduncle incisions, and to anchor the cardiac output probe lead and the cannulae to the skin. After surgery, dogfish were placed into individual flow-through opaque acrylic or wooden boxes and left to recover for 24 h before experimentation.

Experimental protocol

Series 1: the effects of exogenous dogfish angiotensin II on cardiovascular function and plasma catecholamine levels

Dogfish were monitored during an initial period of 30–60 min to assess the stability of P_{CA} and Q traces. Upon stabilisation, control baseline parameters were recorded for 5 min, after which dogfish were given a bolus injection (0.5 ml) of dogfish (*Triakis scyllia*) [Asn^1, Pro^3, Ile^5]-Ang II (dfAng II; Peptide Institute, Osaka) through the caudal vein cannula over a period of 30 s. Cardiovascular responses to the injection were monitored continuously, and following recovery of cardiovascular variables to control baseline levels for a 1-h period, a second dose of dfAng II was injected. Repeating this protocol, five doses of dfAng II (30, 100, 300, 600, 1200 pmol kg^{-1}) and saline were randomly and sequentially tested on each fish ($n = 6$). Each injection was followed by 0.4 ml of saline to clear the cannula.

Using the same experimental protocol as above, three doses of dfAng II (30, 300, and 1200 pmol kg^{-1}) and saline were tested in a second group of dogfish ($n = 6$), first treated with the α-Adrenoceptor blocker, yohimbine (RBI, Natick, Mass.). α-Adrenergic blockade was achieved by slowly (over a 5-min period) injecting a 4-mg kg^{-1} dose of yohimbine 60 min prior to experimentation. Yohimbine was dissolved in 200 µl of ethanol and diluted in saline (4 mg ml^{-1}) prior to injection (1 ml kg^{-1}). Before yohimbine treatment and after the three Ang II doses, the effectiveness of the α-adrenergic blockade was assessed by injection of a catecholamine cocktail (0.1 ml kg^{-1}) prepared in dogfish saline and consisting of 1×10^{-4} mol·l^{-1} adrenaline bitartrate and noradrenaline bitartrate (Arterenol, Sigma).

In both groups of fish, a blood sample (0.35 ml) was taken during the 5-min control period before each dfAng II dose to assess basal plasma catecholamines. Four additional blood samples (0.35 ml) were then taken 1, 2.5, 5 and 10 min after each Ang II injection to assess the effects of dfAng II on circulating plasma catecholamine concentrations. Each blood sample, which was replaced by an equivalent volume of saline, was collected in a 1.5-

ml microcentrifuge tube, and centrifuged immediately at 10,000 *g* for 15 s. The separated plasma was combined with 10 µl of a 5% EDTA/10% sodium bisulphite solution, quick frozen in liquid nitrogen, and stored at −80 °C for later analysis of catecholamines.

The selection of yohimbine as an α-adrenoceptor antagonist in this study was based on preliminary experiments which assessed the effectiveness of yohimbine (4 mg kg^{-1}), prazosin (4 mg kg^{-1}), and phentolamine (3 mg kg^{-1}; RBI, Natick, Mass.) in providing chronic (≥2 h) α-adrenergic blockade against exogenous catecholamine injections. Relative to yohimbine, phentolamine and prazosin were both ineffective in providing the sustained blockade required for chronic experiments. Although yohimbine is an α_2-antagonist in mammals, this adrenoceptor antagonist is known to block the α-adrenoceptors which mediate contraction in the systemic vasculature of fish (Holmgren and Nilsson 1974; Wood and Shelton 1980).

Series 2: the effects of hypotension on circulating Ang II and catecholamine concentrations

To investigate the relative contributions of circulating catecholamines and Ang II to cardiovascular control in hypotensive *S. acanthias*, three separate groups of fish were given one of the following treatments: papaverine; lisinopril + papaverine; or yohimbine + papaverine.

Once the stability of P_{CA} and Q traces had been established, control baseline cardiovascular parameters were recorded for 10 min and fish ($n = 6$) were then injected (0.375 ml kg^{-1}) over the subsequent 10 min period with the smooth muscle relaxant, papaverine (10 mg kg^{-1}; RBI). The papaverine injection was followed by 0.4 ml of saline to clear the caudal vein cannula, and the cardiovascular effects of this hypotensive treatment were then monitored continuously for the following 50 min. A blood sample (0.5 ml) was taken immediately before the papaverine injection, as well as 10, 15, 20, 25, 30, 40, 50 and 60 min after the beginning of the injection for subsequent analysis of catecholamines and [Asn^1, Pro^3, Ile^5]-Ang II. Each blood sample was replaced by an equivalent volume of saline, and plasma was obtained by centrifugation and treated as above (see Series 1) prior to being frozen and stored at −80 °C.

In the lisinopril + papaverine or yohimbine + papaverine treatments, the cardiovascular effects of papaverine were assessed 1 h after dogfish received an injection of either the angiotensin converting enzyme (ACE) inhibitor, lisinopril (Sigma), or yohimbine (see series 1). Blockade of the RAS was achieved by injecting a 10^{-4} mol kg^{-1} dose of lisinopril (1 ml kg^{-1}) over a 2 min period. In both treatments, the cardiovascular effects of the blockers were monitored, and blood samples (0.5 ml) were taken before (0 min and 5 min) and after (5, 10, 15, and 20 min) injection of either lisinopril or yohimbine to assess their effects on circulating concentrations of catecholamines and dfAng II.

In the lisinopril + papaverine treatment, the effectiveness of ACE blockade was assessed by comparing the cardiovascular effects of a bolus injection of 1000 pmol kg^{-1} [Ans^1, Val^5, Asn^9]-Ang I (salmon Ang I; Sigma) given before and after lisinopril. Salmon Ang I was used to test the effectiveness of ACE blockade because of the unavailability of dogfish Ang I at the time of experimentation.

Analytical procedures

P_{CA} was measured with a UFI model 1050BP (UFI, Morro Bay, Calif.) pressure transducer that was calibrated against a static water column. Mean blood pressure was calculated as: [systolic pressure + 2(diastolic pressure)]/3. The perivascular flow probes used to measure Q were connected to a Transonic T106 small animal blood flow meter (Transonic Systems, Ithaca, NY). These probes were precalibrated in the factory and verified in the laboratory by pump perfusion of the heart of an immersed euthanized fish with saline at known flow rates. Both P_{CA} and Q signals were recorded with a data acquisition system (Biopac System, Goleta, Calif.) and collected at 0.04 s intervals using Acknowledge III (Biopac System) data acquisition software. In the absence of central venous pressure measurements, systemic vascular resistance (R_S) was calculated as mean P_{CA} divided by Q (i.e. $R_S = P_{CA}/Q$). This calculation is valid assuming that venous pressure is close to zero and therefore without significant effect on R_S. Although some of the treatments in the present study may have altered venous pressure, potential errors in the calculation of R_S are assumed to be small because of the predominant contribution of the systemic pressure in setting R_S. Heart rate (f_H) was derived from the caudal artery pressure pulse trace, and stroke volume (V_S) was calculated as Q divided by f_H (i.e. $V_S = Q/f_H$).

Plasma catecholamines (adrenaline and noradrenaline) were determined on alumina-extracted plasma samples (0.2 ml) using high-pressure liquid chromatography (HPLC) with electrochemical detection (Bernier and Perry 1997). The extracted samples were passed through an Ultratechsphere ODS-C18 5 µm column (HPLC Technology), using a catecholamine and metanephrine mobile phase (Chromosystems, Munich, Germany). The separated amines were integrated using the Star Chromatography software program (version 4.0, Varian, Walnut Creek, Calif.). Concentrations were calculated relative to appropriate standards and with 3,4-dihydroxybenzylamine hydrobromide (DHBA) as an internal standard in all determinations. Plasma Ang II concentrations were determined on extracted plasma samples and measured by radioimmunoassay (Bernier et al. 1999). The antiserum used in this assay was initially raised against mammalian Ang II ([Asp^1, Ile^5]-Ang II; Yamaguchi 1981). Relative to the standard curve obtained with [Asp^1, Ile^5]-Ang II (Sigma), the antiserum exhibited 73.4% and 0.08% cross-reactivity with dogfish [Asn^1, Pro^3, Ile^5]-Ang II and dogfish [Asn^1, Pro^3, Ile^5, Gln^9]-Ang I, respectively (Bernier et al. 1999). The dilution curve of immunoreactive Ang II in extracted *S. acanthias* plasma was parallel to the standard curve of [Asn^1, Pro^3, Ile^5]-Ang II. The intra- and inter-assay coefficients of variation were 5.5% and 8.1%, respectively.

Statistical analyses

Data are presented as mean values ± 1 SEM. In general, the statistical significance of the observed effects of an injection within a group was tested using a one-way repeated-measures analysis of variance (ANOVA). However, when the criteria for parametric analysis were violated (unequal variance or lack of normal distribution) a non-parametric one-way repeated-measures ANOVA on ranks was used to determine the observed effects of an injection on these parameters. Dunnett's *post-hoc* multiple-comparison test was used to compare the pre-injection control data point with values at subsequent times. Following a given dfAng II injection, differences between the control and maximum plasma catecholamine levels were analysed using a Student's paired *t*-test. Among the various dfAng II injections within a treatment, differences in the effects on cardiovascular variables, or differences in the maximum increase of a catecholamine, were determined using one-way ANOVA followed by a Tukey test for multiple comparisons. The statistical significance of observed differences between the means of two treatments was determined by a Student's *t*-test. For a given parameter and time, differences among the 3 papaverine treatments were assessed with a one-way ANOVA followed by Dunnett's multiple-comparisons test to compare the lisinopril + papaverine and the yohimbine + papaverine treatments to the control papaverine treatment. Throughout the paper, percent changes were calculated as: [(mean response − mean control value)/mean control value] × 100. The significance level for all statistical tests was $P < 0.05$.

Results

Series 1: the effects of exogenous dfAng II on cardiovascular function and plasma catecholamine levels

Bolus injections of dfAng II between 30 pmol $kg^{-1}$and 1200 pmol kg^{-1} elicited increases in P_{CA} and R_S, and decreases in Q, f_H, and V_S (Table 1; Fig. 1A–E). While

Table 1 Absolute changes in cardiovascular parameters following bolus injection of saline or [Asn^1, Pro^3, Ile^5]-Ang II in control or yohimbine-treated spiny dogfish, *Squalus acanthias*. Values are means ±1 SEM. Values in parentheses represent *n* values. f_H heart rate (beats min^{-1}), P_{CA} mean caudal artery pressure (kPa), Q cardiac output (ml min^{-1} kg^{-1}), R_S systemic resistance (kPa ml^{-1} min^{-1} kg^{-1}), V_S stroke volume (ml kg^{-1} beat^{-1})

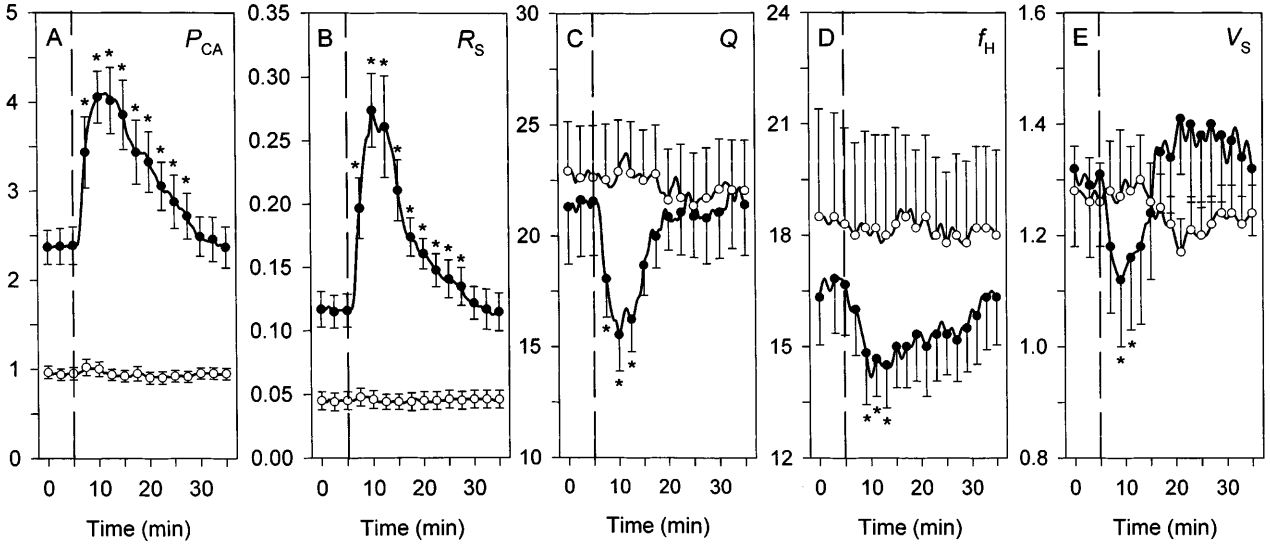
[Asn^1, Pro^3, Ile^5]-Ang II dose (pmol kg^{-1})						
Parameter	Saline	30	100	300	600	1200
Control treatment (6)						
P_{CA}	+0.44 ± 0.06^{a}	+1.02 ± 0.23*^{ab}	+1.43 ± 0.07*^{bc}	+1.91 ± 0.14*^{c}	+2.15 ± 0.19*^{c}	+2.09 ± 0.26*^{c}
R_S	+0.02 ± 0.00^{a}	+0.08 ± 0.01*^{ab}	+0.12 ± 0.02*^{abc}	+0.18 ± 0.02*^{bcd}	+0.22 ± 0.03*^{cd}	+0.25 ± 0.04*^{d}
Q	+1.7 ± 0.6^{a}	−4.8 ± 1.1*^{b}	−5.6 ± 1.6*^{b}	−6.7 ± 1.6*^{b}	−7.3 ± 1.7*^{b}	−8.1 ± 1.3*^{b}
f_H	+0.8 ± 0.6^{a}	−3.7 ± 1.1*^{b}	−3.3 ± 0.8*^{b}	−3.5 ± 0.8*^{b}	−3.7 ± 1.0*^{b}	−4.7 ± 0.8*^{b}
V_S	+0.08 ± 0.06^{a}	−0.12 ± 0.05^{ab}	−0.25 ± 0.05*^{b}	−0.26 ± 0.06*^{b}	−0.29 ± 0.05*^{b}	−0.32 ± 0.06*^{b}
Yohimbine treatment (6)						
P_{CA}	+0.17 ± 0.07	+0.17 ± 0.09		+0.12 ± 0.03		+0.25 ± 0.14
R_S	+0.01 ± 0.00	+0.01 ± 0.00		+0.01 ± 0.00		+0.01 ± 0.00
Q	+2.7 ± 0.5	+4.3 ± 2.0		+2.0 ± 1.2		+1.1 ± 0.4
f_H	+0.2 ± 0.5	+0.8 ± 0.5		−0.8 ± 0.9		−0.7 ± 1.0
V_S	+0.02 ± 0.07	−0.09 ± 0.07		−0.01 ± 0.08		+0.07 ± 0.09

* Significantly different from resting value for a given injection. Means that do not share a common letter for a given parameter and treatment are significantly different from each other ($P < 0.05$)

the dfAng II-elicited increase in P_{CA} was dose-dependent between 30 pmol kg^{-1} and 300 pmol kg^{-1}, the increase in R_S was dose-dependent between 30 pmol kg^{-1} and 1200 pmol kg^{-1} (Table 1). The dfAng II injections also elicited dose-dependent increases in plasma noradrenaline (Fig. 2A) and adrenaline (Fig. 2B) levels. For a given Ang II dose (30–1200 pmol kg^{-1}), the increase in plasma adrenaline was significantly greater (2–2.7 times) than the increase in noradrenaline (Fig. 2A–B).

Fig. 1 **A** Mean caudal artery pressure (P_{CA}, kPa), **B** systemic vascular resistance (R_S, kPa ml^{-1} min^{-1} kg^{-1}), **C** cardiac output (Q, ml min^{-1} kg^{-1}), **D** heart rate (f_H, beats min^{-1}) and **E** stroke volume (V_S, ml kg^{-1} beat^{-1}) in intact control ($n = 6$; *filled circles*) and yohimbine-treated ($n = 6$; *unfilled circles*) Pacific spiny dogfish, *Squalus acanthias*, given a bolus injection of 300 pmol kg^{-1} homologous [Asn^1, Pro^3, Ile^5]-Ang II. The *dashed line* in each graph indicates the time of Ang II injection. An *asterisk* denotes a significant difference from the resting value immediately preceding the dashed line ($P < 0.05$). Values are means ±1 SEM

In comparison to the control group, fish treated with the α-adrenergic antagonist yohimbine had significantly lower resting mean P_{CA} and R_S, similar Q (Table 2; Fig. 1A–C), and higher basal plasma noradrenaline and adrenaline concentrations (Fig. 2A–B). Relative to the pre-injection control condition, yohimbine treatment reduced the pressure response and the increase in R_S to a bolus injection of catecholamines by 81% and 93%, respectively, and abolished the reduction in Q (Table 2). Although bolus injections of dfAng II (30–1200 pmol kg^{-1}) had no significant effect on any of the measured cardiovascular parameters in the yohimbine-treated dogfish (Table 1; Fig. 1A–E), they elicited dose-dependent (30–1200 pmol kg^{-1}) increases in plasma catecholamine levels (Fig. 2A–B). As in the control group, the increases in plasma adrenaline were significantly greater (2–2.5 times) than the increases in noradrenaline (Fig. 2A–B).



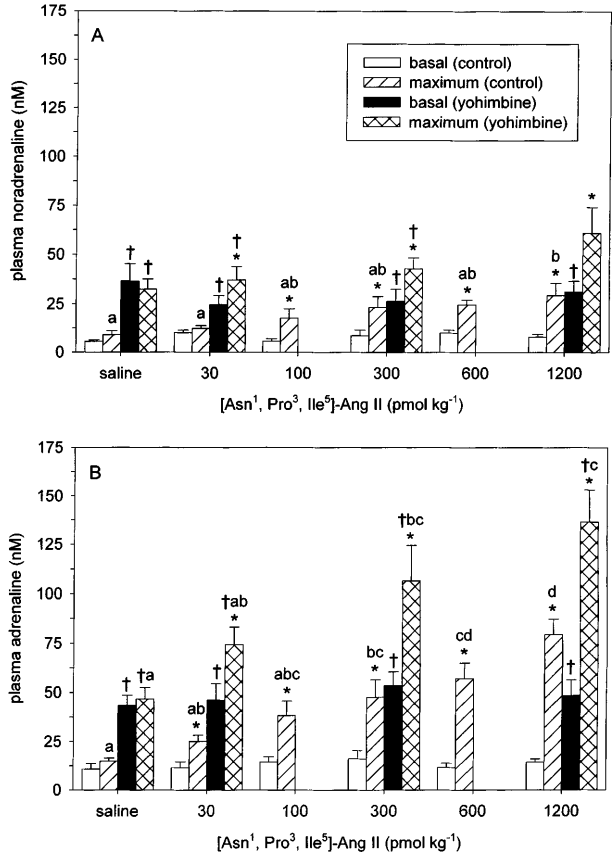


Fig. 2 Effects of bolus injections of either saline or homologous [Asn[1], Pro[3], Ile[5]]-Ang II on plasma **A** noradrenaline and **B** adrenaline concentrations in intact control ($n = 6$) and yohimbine-treated ($n = 6$) Pacific spiny dogfish, *S. acanthias*. The *unfilled bars* and *black bars* indicate the basal catecholamine concentrations prior to the injection in the control and yohimbine-treated dogfish, respectively. The *diagonal-hatched bars* and *cross-hatched bars* indicate the maximum catecholamine concentrations in response to the injection in the control and yohimbine-treated dogfish, respectively. An *asterisk* denotes a significant difference from the basal value for a given dose and treatment. Means that do not share a common letter for a given parameter and treatment are significantly different from each other. † denotes a significant difference from the corresponding value in the control treatment for a given dose ($P < 0.05$). Values are means ±1 SEM

Injection of saline alone had no effect on the resting cardiovascular parameters or on the basal plasma catecholamine concentrations of either the control or yohimbine-treated dogfish (Table 1, Fig. 2A–B).

Series 2: the effects of hypotension on circulating Ang II and catecholamine concentrations

Intravenous injection of papaverine elicited rapid and transient decreases in P_{CA} (47%) and R_S (39%), and no significant change in Q (Fig. 3A–C), f_H, or V_S (data not shown). Papaverine treatment elicited sustained increases in plasma adrenaline, noradrenaline and Ang II concentrations (Fig. 3D–E).

Lisinopril administration produced a small and transient decrease in P_{CA} (17%), and no significant change in R_S, Q, plasma catecholamines, or plasma Ang II (Fig. 4A–E). In comparison to control fish, lisinopril treatment abolished the changes in P_{CA}, R_S, and Q associated with a bolus injection of [Asn[1], Val[5], Asn[9]]-Ang I (Table 3) thus confirming ACE inhibition. Papaverine injection in lisinopril-treated fish elicited rapid and sustained decreases in P_{CA} (64%) and R_S (56%), and no significant change in Q (Fig. 4F–H), f_H, or V_S (data not shown). The papaverine-elicited decreases in P_{CA} and R_S were significantly greater in the lisinopril-treated dogfish than in the control papaverine group. Papaverine injection in lisinopril-treated fish also produced a transient increase in plasma adrenaline, a small and sustained increase in plasma noradrenaline, and had no effect on plasma Ang II concentrations (Fig. 4I–J).

Yohimbine administration produced marked and sustained decreases in P_{CA} (56%) and R_S (54%), no significant change in Q, and increases in plasma catecholamine and Ang II concentrations (Fig. 5A–E). Papaverine injection in yohimbine-treated fish resulted in further decreases in P_{CA} (47%), and R_S (27%), as well as decreases in Q (24%; Fig. 5F–H), and V_S, but no significant change in f_H (data not shown). In comparison to the pre-yohimbine baseline cardiovascular variables, the combined yohimbine + papaverine treatment decreased P_{CA} and R_S by 72% and 61%, respectively. The papaverine-elicited decreases in P_{CA} and R_S in the yohimbine-treated dogfish were significantly greater than in the control papaverine group. Papaverine injection in α-adrenoceptor blocked dogfish also resulted in plasma

Table 2 The effects of a bolus injection of catecholamines on the cardiovascular variables of intact control and yohimbine-treated spiny dogfish, *S. acanthias*. The catecholamine injection contained 10 nmol kg^{-1} body wt noradrenaline bitartrate and adrenaline bitartrate. Values are mean ±1 SEM

Treatment	n	P_{CA} (kPa)	R_S (kPa ml^{-1} min^{-1} kg^{-1})	Q (ml min^{-1} kg^{-1})
Control	6			
Basal values		2.70 ± 0.26	0.11 ± 0.01	26.1 ± 3.3
Post-injection		3.96 ± 0.33*	0.30 ± 0.06*	15.8 ± 3.5*
Change from basal		1.26 ± 0.19	0.19 ± 0.05	−10.2 ± 2.1
Yohimbine	6			
Basal values		1.15 ± 0.19†	0.05 ± 0.01†	23.0 ± 2.7
Post-injection		1.39 ± 0.24*	0.07 ± 0.01*	19.9 ± 1.5
Change from basal		0.25 ± 0.10#	0.01 ± 0.00#	−3.1 ± 1.9#

* Significantly different from basal value for a given treatment
† Significantly different from basal value of the control treatment
# Significantly different from the change from basal value of the control treatment ($P < 0.05$)

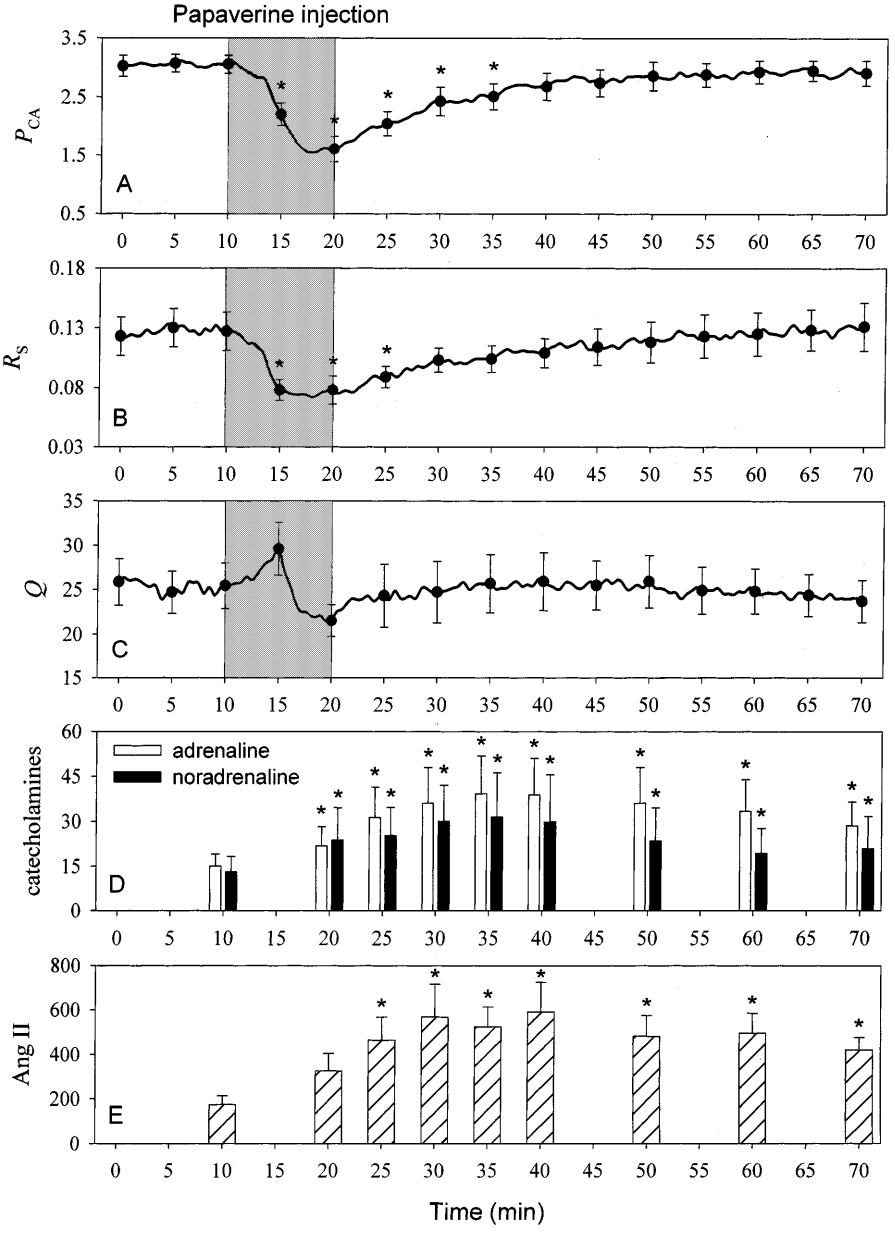


Fig. 3 Effects of an intravenous injection of the smooth muscle relaxant, papaverine (10 mg kg^{-1}) on **A** P_{CA} (kPa), **B** R_S (kPa ml^{-1} min^{-1} kg^{-1}), **C** Q (ml min^{-1} kg^{-1}), **D** plasma catecholamines (nM) and **E** plasma [Asn^1, Pro^3, Ile^5]-Ang II (pM) in Pacific spiny dogfish, *S. acanthias* ($n = 6$). The time during which papaverine was injected is shown by a *gray box* in graphs **A**, **B**, and **C**. An *asterisk* denotes a significant difference from the control value immediately preceding the papaverine injection (10 min) for a given parameter ($P < 0.05$). Values are means ±1 SEM

catecholamine and Ang II concentrations that were significantly higher than in the control papaverine group (Fig. 5I–J).

The overall relationship between plasma [Asn^1, Pro^3, Ile^5]-Ang II and plasma catecholamine (adrenaline + noradrenaline) concentrations at each sampling time in the papaverine, lisinopril + papaverine, and yohimbine + papaverine treatments is shown in Fig. 6. This relationship ($r^2 = 0.61$) is significant ($P < 0.001$), and can be described by the following linear equation: [catecholamines] = 0.047[Ang II] + 34.57, where [catecholamines] is in nM and [Ang II] is in pM.

Discussion

Although previous studies have demonstrated convincingly that the RAS is activated (Galli and Kiang 1990) and plays a role in blood pressure regulation (Hazon et al. 1989) in elasmobranchs during hypotension, they were not designed to distinguish between the *direct* versus the *indirect* effects of the RAS in cardiovascular control. In this study, by simultaneously assessing plasma Ang II, catecholamines and cardiovascular parameters, we have provided evidence that the RAS *indirectly* contributes to blood pressure regulation in *S. acanthias* during hypotension by activation of the humoral adrenergic system.

Reliability of the cardiovascular measurements

The Q and mean arterial blood pressure recorded from resting animals prior to experimentation were comparable to those previously reported in *S. acanthias* (Butler

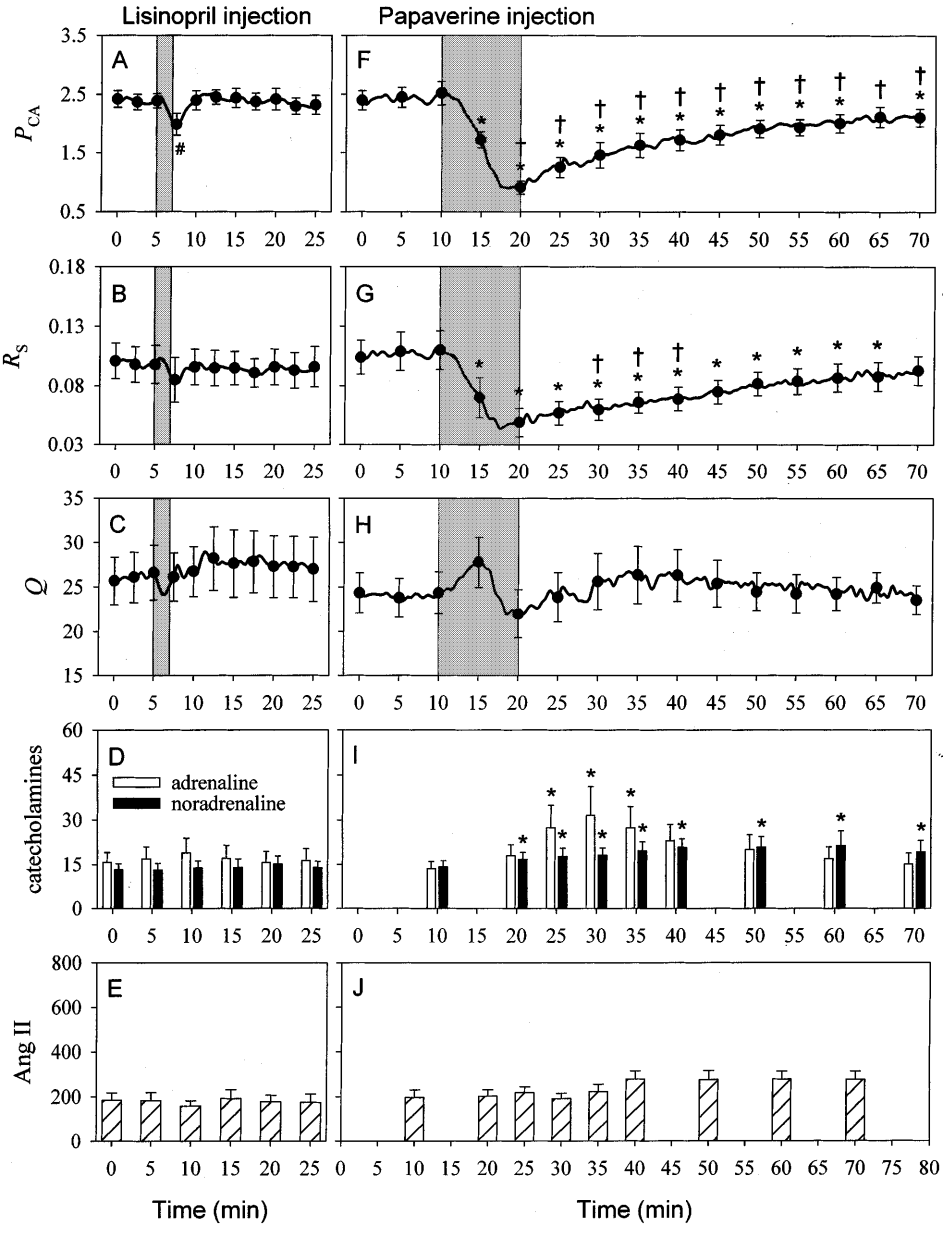


Fig. 4 Effects of an intravenous injection of the angiotensin-converting-enzyme inhibitor, lisinopril (**A**–**E**; 10^{-4} mol kg^{-1}) followed by an intravenous injection of the smooth muscle relaxant, papaverine (**F**–**J**; 10 mg kg^{-1}) on (**A** and **F**) P_{CA} (kPa), (**B** and **G**) systemic vascular resistance R_S (kPa ml^{-1} · min^{-1} kg^{-1}), (**C** and **H**) Q (ml min^{-1} kg^{-1}), (**D** and **I**) plasma catecholamines (nM) and (**E** and **J**) plasma [Asn^1, Pro^3, Ile^5]-Ang II (pM) in Pacific spiny dogfish, *S. acanthias* (n = 6). The time during which lisinopril was injected is shown by a *gray box* in graphs **A**, **B**, and **C**. The time during which papaverine was injected is shown by a *gray box* in graphs **F**, **G**, and **H**. An *asterisk* denotes a significant difference from the control value immediately preceding the papaverine injection (10 min) for a given parameter. † denotes a significant difference from the corresponding value in the papaverine treatment (Fig. 3) for a given parameter. # denotes a significant difference from the control value immediately preceding the lisinopril injection (5 min) for a given parameter ($P < 0.05$). Values are means ±1 SEM

and Metcalfe 1988; Farrell and Jones 1992). However, the resting f_Hs were lower and the V_Ss higher when compared with other reported values for *S. acanthias* (Butler and Metcalfe 1988; Farrell and Jones 1992). Although the physiolgical role of the rigid pericardium in the events of the cardiac cycle of elasmobranchs is equivocal (Lai et al. 1990, 1996; Franklin and Davie 1993), sectioning of the pericardium and placement of the ultrasonic flow probe around the conus arteriosus may have contributed to the lower f_Hs and higher V_Ss recorded in this study. Although absolute blood flow values are reported in this study, we recognize that certain methodological constraints (sectioning of the pericardium, placement of the probe around the contracting conus and highly pulsatile flow) may have led to measurement error. However, the conclusions of this study are based on relative blood flow changes within individual fish and thus, any potential error in the measurement is common to all experiments.

The effects of exogenous dfAng II on cardiovascular function and plasma catecholamine levels

In support of previous observations using a variety of heterologous Ang I and Ang II peptides (Opdyke and Holcombe 1976; Khosla et al. 1983), we found no evidence that physiological doses of dfAng II can directly affect the primary contributors to blood pressure regulation, R_S and Q. In fact, the dose-dependent dfAng II-elicited pressor response appears to be mediated by an increase in R_S that can be ascribed to a dose-dependent increase in humoral catecholamines. α-Adrenoceptor blockade, in addition to eliminating the vasopressor

Table 3 The effects of a bolus injection of [Asn^1, Val^5, Asn^9]-Ang I on the cardiovascular variables of intact control and lisinopril-treated spiny dogfish, *S. acanthias*. The angiotensin injection contained 1000 pmol kg^{-1} body wt [Asn^1, Val^5, Asn^9]-angiotensin I. Values are mean ±1 SEM

Treatment	*n*	P_{CA} (kPa)	R_S (kPa ml^{-1} min^{-1} kg^{-1})	Q (ml min^{-1} kg^{-1})
Control	6			
Basal values		2.42 ± 0.11	0.09 ± 0.01	28.1 ± 3.3
Post-injection		2.89 ± 0.17*	0.13 ± 0.02*	25.1 ± 2.9*
Change from basal		0.47 ± 0.07	0.03 ± 0.01	−3.0 ± 0.8
Lisinopril	6			
Basal values		2.34 ± 0.15	0.10 ± 0.01	25.7 ± 2.5
Post-injection		2.50 ± 0.14	0.11 ± 0.01	25.7 ± 2.6
Change from basal		0.17 ± 0.09$^{\#}$	0.01 ± 0.00$^{\#}$	0.0 ± 0.5$^{\#}$

* Significantly different from basal value for a given treatment
$^{\#}$ Significantly different from the change from basal value of the control treatment ($P < 0.05$)

effects of exogenous dfAng II, was characterised by a drop in R_S despite a five-fold increase in plasma Ang II concentrations. Hence in *S. acanthias*, independent of dosage or amino acid sequence, the cardiovascular effects of exogenous Ang II are mediated indirectly via a stimulation of catecholamine release and their

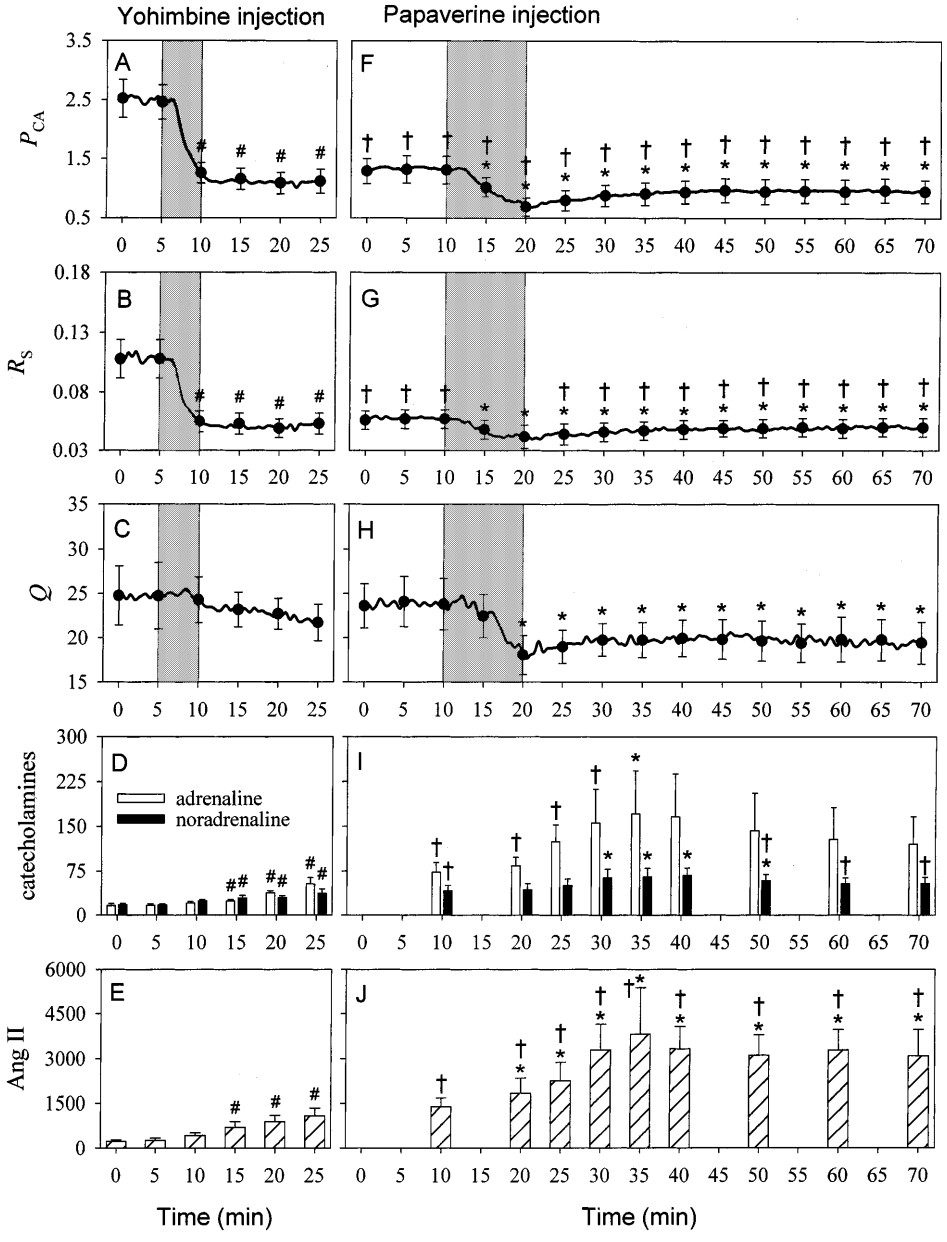


Fig. 5 Effects of an intravenous injection of the α-adrenoceptor antagonist, yohimbine (**A**–**E**; 4 mg kg^{-1}) followed by an intravenous injection of the smooth muscle relaxant, papaverine (**F**–**J**; 10 mg kg^{-1}) on (**A** and **F**) P_{CA} (kPa), (**B** and **G**) R_S (kPa ml^{-1} min^{-1} kg^{-1}), (**C** and **H**) Q (ml min^{-1} kg^{-1}), (**D** and **I**) plasma catecholamines (nM) and (**E** and **J**) plasma [Asn^1, Pro^3, Ile^5]-Ang II (pM) in Pacific spiny dogfish, *S. acanthias* ($n = 6$). The time during which yohimbine was injected is shown by a *gray box* in graphs **A**, **B**, and **C**. The time during which papaverine was injected is shown by a *gray box* in graphs **F**, **G**, and **H**. An *asterisk* denotes a significant difference from the control value immediately preceding the papaverine injection (10 min) for a given parameter. † denotes a significant difference from the corresponding value in the papaverine treatment (Fig. 3) for a given parameter. # denotes a significant difference from the control value immediately preceding the yohimbine injection (5 min) for a given parameter ($P < 0.05$). Values are means ±1 SEM

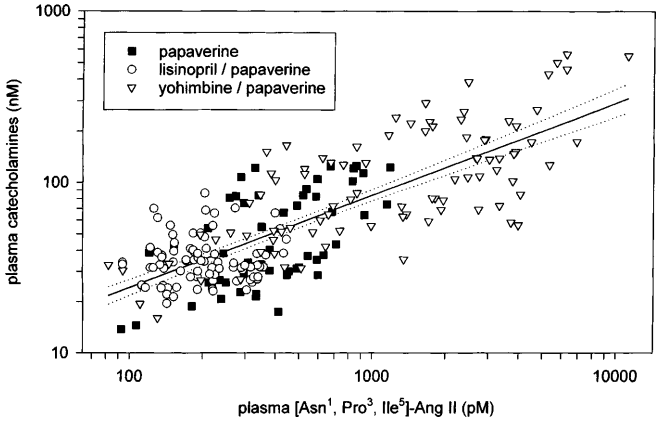


Fig. 6 Correlation between plasma [Asn[1], Pro[3], Ile[5]]-Ang II and total plasma catecholamines in Pacific spiny dogfish, *S. acanthias*, before and after receiving an intravenous injection of either papaverine alone (*filled square*), an injection of lisinopril followed by papaverine (*unfilled circle*), or an injection of yohimbine followed by papaverine (*unfilled triangle*). Linear regression relationship ($r^2 = 0.61$) and its 95% confidence interval are indicated by the *solid* and *dotted lines*, respectively

subsequent interaction with the α-adrenoceptors of the vasculature.

These results are in sharp contrast with the direct and adrenergically independent dfAng II-induced pressor response observed in *Scyliorhinus canicula* (Tierney et al. 1997). Although the amino acid sequence of dogfish angiotensin is unique (Takei et al. 1993), differences between the sequence of homologous and heterologous angiotensins cannot account for the discrepant results obtained in *S. canicula* (Tierney et al. 1997) versus *S. acanthias* (Opdyke and Holcombe 1976; Khosla et al. 1983). Similarly, Ang II dosage differences cannot explain the different mode of action of Ang II in *S. canicula* and *S. acanthias*. Although the original studies in *S. acanthias* used supraphysiological doses of Ang II (Opdyke and Holcombe 1976; Khosla et al. 1983), in the present study we used physiological doses similar to those used by Tierney et al. (1997) in *S. canicula*. Hence, differences in the mode of action of Ang II in *S. acanthias* and *S. canicula* may reflect true species differences. For example, although α-adrenoceptor blockade inhibits the pressor response to exogenous catecholamines for at least 90 min in both *S. canicula* (Tierney et al. 1997) and *S. acanthias* (this study), the cardiovascular effects of α-adrenoceptor blockade appears to differ between these two dogfish species. Whereas blood pressure recovered within 15 min after an injection of phentolamine in *S. canicula* (Tierney et al. 1997), P_{CA} and R_S remained depressed for hours in yohimbine-treated *S. acanthias* despite an increase in plasma Ang II. Further investigations into the pharmacology of α-adrenoceptors, and experiments aimed at determining the presence or absence of functional vascular Ang II receptors in both species, may help to resolve whether Ang II has a single or dual mode of action in elasmobranchs.

Although the failure of heterologous Ang II to directly constrict blood vessels in *S. acanthias* has previously been interpreted as evidence that Ang II vascular receptors are absent in dogfish (Opdyke and Holcombe 1976; Carroll 1981), there is recent evidence to the contrary (Hamano et al. 1998). Whereas a pharmacological dose of 780 000 pM of teleost Ang II failed to constrict isolated rings from the coeliac artery in *S. acanthias* (Carroll 1981), a lower dose of 100 000 pM of dfAng II elicited α-adrenoceptor-independent vasopressor activity on the same vessel in *Triakis scyllia* (Hamano et al. 1998). Given the known differences in the vasopressor activity of homologous versus heterologous Ang II in dogfish (Takei et al. 1993; see also below), these results suggest that the failure of previous experiments to demonstrate Ang II vascular receptor in *S. acanthias* may be explained by their selection of peptide dosage. While the results of Hamano et al. (1998) provide evidence for functional low-affinity Ang II vascular receptors in *large arteries* of dogfish, our results do not provide any physiological evidence for the involvement of Ang II receptors in the *resistance vessels* of *S. acanthias*.

Although homologous and heterologous angiotensins appear to have the same indirect cardiovascular effects in *S. acanthias*, the important amino acid sequence differences between these peptides (Takei et al. 1993) are responsible for differences in their vasopressor activity. As previously observed in *Triakis scyllia* with dfAng I (Takei et al. 1993), dfAng II had a much greater vasopressor activity than heterologous Ang II in *S. acanthias* (Opdyke and Holcombe 1976; Khosla et al. 1983). Conversely, the dose-response relationship for the vasopressor effects of dfAng I in *T. scyllia* (Takei et al. 1993), dfAng II in *S. canicula* (Tierney et al. 1997) and dfAng II in *S. acanthias* are remarkably similar.

In contrast to the equal potency of teleost, tetrapod, and mammalian Ang II agonists in stimulating catecholamine release in rainbow trout (Bernier and Perry 1997), it appears that the specific amino acid sequence of the Ang II injected in dogfish may impact on its ability to elicit catecholamine release. In elasmobranchs, the catecholamine contents of the most important site of catecholamine storage, the axillary bodies, are composed of approximately 80% noradrenaline and 20% adrenaline (Abrahamsson 1979). Moreover, storage granules of both catecholamines have been identified and these appear in separate cells (Coupland 1971). In *S. acanthias*, since Ang II elicits catecholamine release in the presence of the ganglionic blocker hexamethonium, the stimulatory effects of Ang II on the chromaffin tissue are thought to be direct (Opdyke and Holcombe 1976). However, while Opdyke et al. (1981) reported a preferential increase in plasma noradrenaline over adrenaline in response to supraphysiological doses of heterologous Ang II (4800 pmol kg^{-1}), opposite effects were obtained with physiological doses (30–1200 pmol kg^{-1}) of dfAng II in this study. Given the differential haemodynamic responses to adrenaline and noradrenaline in *S. acan-*

thias (Capra and Satchell 1977a), the preferential stimulation of adrenaline by dfAng II may be relevant to the role of the RAS in cardiovascular control (i.e. elevation of blood pressure and blood volume). For example, in dogfish, adrenaline is a more potent vasopressor than noradrenaline whereas the latter is a more potent vasodilator (Capra and Satchell 1977a). The preferential increase in plasma adrenaline over noradrenaline with dfAng II in *S. acanthias* is similar to the stimulatory effects of homologous Ang II in teleosts (Carroll and Opdyke 1982; Bernier and Perry 1997, 1999) and in mammals (Butler et al. 1994).

Series 2: the effects of hypotension on circulating Ang II and catecholamine concentrations

The transient hypotensive effects of papaverine in *S. acanthias* concur with the previously observed effects of this smooth muscle relaxant in *S. canicula* (Hazon et al. 1989) and in a number of teleost species (Nishimura et al. 1979; Tierney et al. 1995; Bernier et al. 1999). The significant and prolonged increases in plasma Ang II concentration following this hypotensive stress provide the first direct evidence that papaverine treatment truly stimulates the RAS in elasmobranchs. These results also corroborate the preliminary observations of Galli and Kiang (1990) showing that a 30% haemorrhage can increase immunoreactive [Val^5]-Ang II in the nurse shark, *Ginglymostoma cirratum*. Because blockade of the RAS exacerbates and prolongs the hypotensive effects of papaverine in *S. acanthias,* as in *S. canicula* (Hazon et al. 1989), the papaverine-elicited increase in plasma Ang II must play a significant role in cardiovascular control during a hypotensive stress. On the other hand, since various ACE inhibitors either have no cardiovascular effect (Opdyke and Holcombe 1976; Hazon et al. 1989) or only a small and brief hypotensive effect (this study), the RAS does not appear to be involved in the cardiovascular control of elasmobranchs during normotensive conditions. In contrast, the RAS plays a tonic anti-depressor role in teleosts and ACE blockade chronically reduces blood pressure (Olson 1992; Tierney et al. 1995; Bernier et al. 1999). Furthermore, while lisinopril treatment has no effect on circulating catecholamines in *S. acanthias*, it is associated with a significant decrease in the basal circulating concentration of adrenaline in *Oncorhynchus mykiss* (Bernier et al. 1999).

Because the humoral adrenergic system appears to be an essential intermediate for the involvement of Ang II in cardiovascular control (see Series I; Opdyke and Holcombe 1976; Khosla et al. 1983), the cardiovascular effects of the RAS in hypotensive *S. acanthias* are likely to be mediated through an increase in plasma catecholamines. Prior to this study, however, the effects of a hypotensive stress on plasma catecholamine levels in elasmobranchs had not been assessed. The significant correlation between plasma Ang II and catecholamine concentrations following the different papaverine treatments suggests that the RAS may play a central role in controlling the recruitment of the adrenergic system in response to hypotension. However, although lisinopril completely blocked the papaverine-elicited increase in plasma Ang II, ACE blockade was associated only with a partial reduction of the overall increase in plasma catecholamines following the hypotensive treatment. Hence, while a portion of the increase in plasma catecholamines may be attributed to an elevation in plasma Ang II, other secretagogues may also be involved in stimulating catecholamine release during a hypotensive stress in dogfish. The presence of cholinergic and non-cholinergic neuronal pathways innervating the chromaffin cells of the axillary bodies (Abrahamsson 1979; Opdyke et al. 1983; Reid et al. 1995) offers a variety of potential mechanisms for the control of catecholamine release in response to stress. Because treatment of dogfish with the ganglionic blocker hexamethonium results in a significant reduction in blood pressure, it appears likely that at least part of the vasomotor tone provided by circulating catecholamines is controlled by cholinergic neuronal activity (Holcombe et al. 1980). In addition to Ang II, potassium (Opdyke et al. 1983), urotensin II (Conlon et al. 1996), and C-type natriuretic peptides (CNP; McKendry et al. 1999) are other non-cholinergic secretagogues that have been shown to stimulate catecholamine release in elasmobranchs. While some of these catecholamine secretagogues may also be involved in cardiovascular regulation (Conlon et al. 1996; McKendry et al. 1999), their specific involvement during a hypotensive stress remains to be ascertained.

In elasmobranchs, the location of the axillary bodies, in the venous blood immediately behind the heart (Abrahamsson 1979), has often been associated with their potential to act as a source of adrenergic cardiac control (for references, see Morris and Nilsson 1994). Although the cardiac responses to catecholamine injections are variable (Capra and Satchell 1977a; Morris and Nilsson 1994), catecholamines generally elicit β-adrenoceptor-mediated positive inotropic and chronotropic effects on the isolated heart of *S. acanthias* (Capra and Satchell 1977b). In this study, however, despite the elevated circulating catecholamine levels associated with a variety of treatments, we did not observe any stimulatory effects in vivo on the heart of *S. acanthias*. While the potential stimulatory effects of the dfAng II-elicited increase in plasma catecholamines might have been masked by vasopressor-mediated reflexive (Lutz and Wyman 1932) or passive inhibition of the heart, the Ang II-elicited increase in plasma catecholamines in the yohimbine-treated dogfish was also without any cardiac effect. By itself, Ang II does not affect the contractility of the isolated dogfish heart (Opdyke et al. 1982). Furthermore, the significant increases in plasma catecholamines elicited by either yohimbine or papaverine treatment were not associated with any change in either f_H or V_S. In fact, in the combined yohimbine + papaverine treatment,

although the post-papaverine circulating concentration of plasma adrenaline reached almost 200 nM, V_S and Q decreased significantly in parallel with the drops in R_S and P_{CA}. Presumably, this reduction in V_S results from a decrease in venous return. In rainbow trout, in contrast, either α-adrenoceptor blockade or the imposition of hypotensive stressors is characterised by a significant increase in Q (Bernier et al. 1999). Hence, in dogfish, while circulating catecholamines may be important in maintaining resting cardiac activity (Short et al. 1977), they do not appear to be involved in increasing Q either at rest or following a hypotensive stress. Similarly, during exposure to hypoxia, the increase in circulating catecholamines observed in *S. canicula* appear to be of little importance in mediating the rise in cardiac V_S (Short et al. 1977). On the other hand, the marked vasodepressor actions of yohimbine and phentolamine (Opdyke et al. 1972; Holcombe et al. 1980) in normotensive *S. acanthias* suggest that circulating catecholamines are an important mediator in the control of basal systemic vascular resistance.

In our ongoing attempt to understand the importance of, and the mechanisms that characterise, the interaction between the RAS and the adrenergic system in the cardiovascular control of fishes, this study provides direct evidence that both systems are essential to cardiovascular homeostasis in hypotensive elasmobranchs. Although the RAS does not appear to be involved in the minute-to-minute regulation of resting blood pressure, the circulating concentrations of plasma Ang II increase in proportion to the intensity of hypotension. However, in *S. acanthias*, irrespective of whether the increase in plasma Ang II has an endogenous or exogenous origin, the cardiovascular effects of dfAng II are mediated indirectly through an interaction with the adrenergic system. A strong correlation between the circulating concentrations of plasma Ang II and catecholamines suggests that the nature of this interaction primarily involves the humoral component of the adrenergic system. Plasma catecholamines, in addition to their tonic role in maintaining basal cardiac activity and vascular resistance, can be recruited by Ang II and other secretagogues during a hypotensive stress and play a key role in the regulation of systemic vascular resistance. On the other hand, the ability of plasma catecholamines to enhance cardiac activity in dogfish appears to be limited.

Acknowledgements We would like to thank all of the staff at Bamfield Marine Station, and especially the Research Coordinator, Joelle Harris, for their hospitality and their assistance. We are also grateful to Dr. K. Yamaguchi for supplying us with the angiotensin II antiserum. This study was supported by the NSERC of Canada operating and equipment grants to SFP. NJB was the recipient of an Ontario Graduate Scholarship. KMG was the recipient of travel awards from the Carnegie Trust for the Universities of Scotland and the *Journal of Experimental Biology*. The animals were cared for in accordance with the principles of the Canadian Council for Animal Care *Guide to the Care and Use of Experimental Animals*, vol 1 (1980) and vol 2 (1984). The experiments comply with the "Principles of animal care" publication number 86-23, revised 1985, of the American National Institute of Health.

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Communicated by I.D. Hume