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Release of purine and pyrimidine nucleosides and their catabolites from the perfused rat hindlimb in response to noradrenaline, vasopressin, angiotensin II and sciatic-nerve stimulation

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Uric acid and uracil were released at constant rates (0.95 and 0.4 nmol/min per g respectively) by the perfused rat hindlimb. Noradrenaline, vasopressin or angiotensin II further increased the release of these substances 2–5-fold, coinciding with increases in both perfusion pressure (vasoconstriction) and O_2 uptake. The hindlimb also released, but in lesser amounts, uridine, hypoxanthine, xanthine, inosine and guanosine, and all but hypoxanthine and guanosine were increased during intense vasoconstriction. Uric acid and uracil releases were increased by noradrenaline in a dose-dependent manner. However, the release of these substances did not fully correspond with the dose-dependent increase in O_2 uptake and perfusion pressure, where changes in the latter occurred at lower doses of noradrenaline. Sciatic-nerve stimulation (skeletal-muscle contraction) did not increase the release of uracil, uric acid or uridine, but instead increased the release of inosine (7-fold) and hypoxanthine (2-fold). Since the UTP content as well as the UTP/ATP ratio are higher in smooth muscle than in skeletal muscle, it is proposed that release of uric acid and uracil arises from increased metabolism of the respective adenosine and uridine nucleotides during intense constriction of smooth muscle.

INTRODUCTION

The measurement of the purine bases hypoxanthine, xanthine, inosine and adenosine in extracellular fluids has been widely used to provide information on changes in intracellular nucleotides. In addition there is considerable interest in the role of adenosine release in the autoregulation of local blood flow, neurohormonal and metabolic responses (e.g. see ref. [1]). Increases in extracellular levels of the purines have been generally found when intracellular levels of ATP have been decreased as a result of energy demand exceeding supply. For example, tissue hypoxia [2–6], heart and tissue ischaemia [2–4,7], tissue damage [3], or indeed strenuous exercise [3], each leads to the release of purine-derived nucleosides. Stimulation of the sympathetic nerve of the heart to increase work has also been reported to increase the release of inosine [8]. Uric acid may accumulate in the extracellular fluid when the concentrations of the precursor purine metabolites hypoxanthine and xanthine rise and sufficient O_2 is available for xanthine oxidase [5].

There has been speculation that purine release by muscle may result from deamination of AMP, where this serves to stabilize the adenylate energy charge or [ATP]/[ADP] ratio. This is proposed to occur during periods of net ATP hydrolysis, by pulling the myokinase reaction ($2ADP \rightarrow ATP + AMP$) toward ATP production [9–12]. The activation of AMP deaminase by decreasing energy charge [10,12] and the initiation of AMP deamination in the highly aerobic fast-twitch red fibres at very high work loads [13] support this proposal.

Uridine, a pyrimidine, is also released in small amounts from tissues when the intracellular [ATP][ADP]/[AMP] ratio is decreased, as in anoxia or ischaemia [3,4]. However, its relationship to the intracellular role of UTP, UDP and UMP is far from clear, and discussion of the origin of extracellular uridine has largely been ignored [3,4].

Recent advances in h.p.l.c. technology with improved columns have now allowed the separation and detection of uracil in extracellular fluid [2] where it was previously masked within an early group of unresolved peaks [3]. This may be the main reason why there have been no reports of uracil release in association with adenosine, inosine, hypoxanthine or uridine. However, uracil is not released by ischaemic cat and rabbit hearts, even when resolution of the early peaks is optimal [2].

Recently we have been studying the actions of vasopressin, angiotensin and noradrenaline as well as perfusion pressure on O_2 uptake by the perfused rat hindlimb [14]. Our findings to date indicate that each of these perturbations leads to an increase in O_2 uptake by the hindlimb, and we have proposed that the increase may be due to contractile work being done by the vascular tissue during constriction [14]. In the present study we report that the vasoconstrictors noradrenaline, angiotensin II and vasopressin each increase the release of uric acid and uracil from the hindlimb. The release of both these substances appears to be tissue-specific, reflecting the change in metabolism/energetics of the vascular tissue during constriction.

EXPERIMENTAL

Materials and animals

Male hooded Wistar rats (180 ± 5 g body wt.) were fed on a commercial diet (Gibson's, Hobart) with water

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ad libitum and were anaesthetized with pentobarbital sodium (5 mg/100 g body wt. intraperitoneally).

Purines, pyrimidines, nucleosides and nucleotides were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals used were of analytical grade.

Hindlimb perfusion

The experimental procedures for erythrocyte-free hindlimb perfusion at 25 °C, for pressure measurement and for the determination of O_2 uptake were as described previously [14] and based on that of Côté *et al.* [15]. Maintaining the perfusion at 25 °C decreased the basal O_2 uptake and allowed adequate O_2 delivery with a non-erythrocyte perfusate. The absence of erythrocytes also eliminated possible contribution of purines from this source. For electrical stimulation, the skin was removed from the hindlimb and the sciatic nerve exposed and cut to allow positioning of the distal end in a suction electrode. The knee was secured by the tibiopatellar ligament, and the Achilles tendon was attached via a stainless-steel wire to a Harvard Apparatus isometric transducer. Tension development was recorded during electrical stimulation (200 ms trains of 100 Hz applied every 2 s; each impulse in the train being of a duration of 0.1 ms) adjusted (15–20 V) to attain full fibre recruitment. Perfusate samples (0.5 ml) were added to 0.1 ml of 2 M-$HClO_4$ on ice, centrifuged at 8000 *g* for 5 min, and 0.4 ml of the supernatant was neutralized with 2.5 M-K_2CO_3. $KClO_4$ was allowed to precipitate at 0 °C and removed by centrifugation at 8000 *g* for 5 min. This supernatant was used directly for h.p.l.c. analysis or stored frozen.

Muscle samples

Thoracic aortas were dissected from anaesthetized rats, cut open longitudinally in ice-cold 0.9% NaCl, and rinsed free of erythrocytes before being frozen in liquid N_2. Muscle samples were powdered under liquid N_2, homogenized in $HClO_4$ and treated as described above for perfusate samples.

Analysis of perfusate and muscle samples by h.p.l.c.

Reverse-phase h.p.l.c. was conducted as described by Wynants *et al.* [2] with a LKB instrument fitted with a model 2141 variable-wavelength monitor set to read simultaneously at 254 and 280 nm. Throughout, 5 μl portions of a standard mixture, containing nine nucleosides and catabolites, were injected. The concentration of each was 10 μM. The injection volumes were always 20 μl for biological samples. Isocratic conditions were used with a Hibar Li Chrosorb Select B column (25 cm; 5 μm particles; Merck, Darmstadt, Germany) at a flow rate of 0.7 ml/min.

Anion-exchange h.p.l.c. was conducted with a Waters Associates instrument fitted with a Serva Si 100:polyol: DEAE column (5 μm particles) and eluted with a linear gradient of 4.5–139 mM-$(NH_4)_2SO_4$ in 10 mM-Tris/acetate, pH 8.0. Detection was a 254 nm, with data acquisition on a Spectra Physics SP1400 computing integrator.

Identification of uracil

Perfusate (100 ml) was collected from a hindlimb during the maximum stimulated O_2-uptake/pressure-development period after infusion of 5 nM-angiotensin II. Five 20 ml batches were then passed through a Sephadex G25M column (200 ml capacity) and eluted with water. The fractions containing the unknown (putatively uracil by reverse-phase h.p.l.c.) were pooled and freeze-dried. The reconstituted material was then further purified by preparative h.p.l.c. with a Waters Associates Accell QMA anion-exchange column (10 mm diam. × 100 mm). The pooled fractions representing a single peak by reverse-phase and cation-exchange (Mono S; Pharmacia) h.p.l.c. were confirmed as uracil by g.l.c./m.s. Authentic uracil (Sigma) was used for comparison. Both the reference material and the unknown were converted into the bistrimethylsilyl derivative by the method of Stetson & Maybaum [16]. A Hewlett–Packard 5890 gas–liquid chromatograph coupled to a Vacuum Generators 7070F mass spectrometer was used. The column was non-polar capillary HP-1 (Hewlett Packard).

RESULTS

Chromatograph (*a*) of Fig. 1 shows reverse-phase h.p.l.c. of the nine standards routinely used. Retention times as well as A_{254}/A_{280} ratios for a total of 21 compounds are shown in Table 1. The combined use of retention time in conjunction with the A_{254}/A_{280} ratio

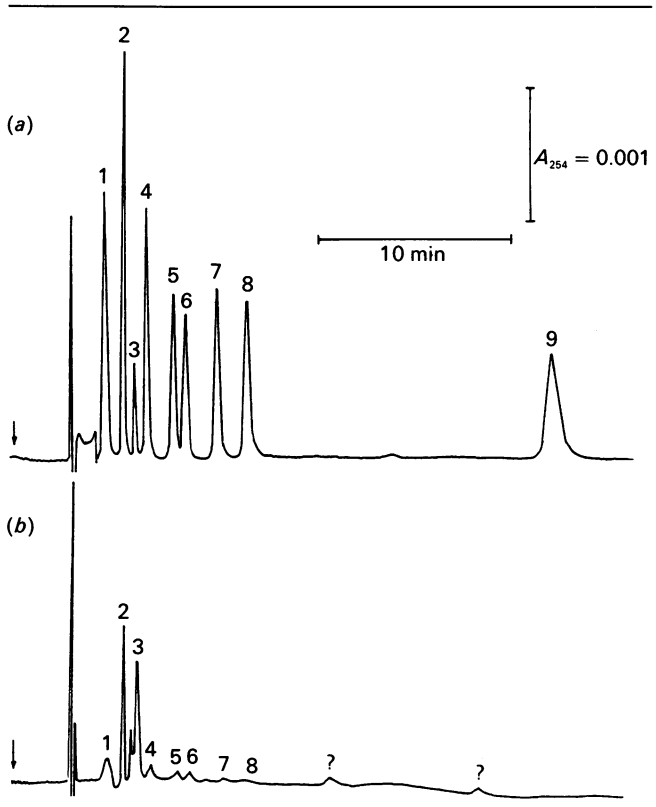

Fig. 1. Reverse-phase h.p.l.c. of standards and of perfusate sample before vasoconstriction or electrical stimulation

The method is given in the Experimental section. Instrument settings were identical for each sample *a* and *b* at 254 nm, 0.005 absorbance units full scale (a.u.f.s.), time constant 0.8 s and spectral band width of 5 nm. (*a*) 50 pmol each of cytidine (1), uracil (2), uric acid (3), uridine (4), hypoxanthine (5), xanthine (6), inosine (7), guanosine (8) and adenosine (9); (*b*) perfusate before vasoconstriction or electrical stimulation (resting state). Sample (*b*) was taken at the time indicated by arrow B in Fig. 3.

Table 1. Retention times and A_{254}/A_{280} ratios for reference purine and pyrimidine nucleotides and their catabolites

Reverse-phase h.p.l.c. was conducted as described in the Experimental section by using a variable-wavelength monitor set to read simultaneously at 254 and 280 nm. The flow rate was 0.7 ml/min.

Reference standard	Retention time (min)	A_{254}/A_{280}
UTP	3.4	2.74
UDP-glucose	3.5	2.75
UDP	3.7	2.70
GTP	4.7	1.68
Pseudouridine	4.7	2.24
Cytidine	4.7	0.65
Orotate	5.0	0.48
$NADP^+$	5.0	4.50
Uracil	5.5	6.00
Uric acid	6.1	0.32
NAD^+	6.2	4.90
ADP	6.4	5.87
Uridine	6.8	2.92
ATP	7.7	6.71
Guanine	7.7	1.24
Hypoxanthine	8.2	11.33
Xanthine	8.8	1.57
Inosine	10.3	5.67
Guanosine	12.0	1.80
Adenine	12.3	4.75
Adenosine	27.7	7.60

was useful in initially identifying peaks where potential overlap was a problem (e.g. in the region of cytidine). In general, the A_{254}/A_{280} ratios of the standards compared closely with those published [17].

Chromatograph (*b*) of Fig. 1 shows that uric acid (peak 3) and uracil (peak 2) were the predominant substances released during basal conditions. Uric acid has a characteristic absorbance spectrum (a low A_{254}/A_{280} ratio) and was therefore readily identified. It has also been reported to be released in small amounts by the perfused heart [2]. Peak 2, putatively identified as uracil, required confirmation, as its presence, particularly in relatively high concentrations, was unexpected. Thus samples containing this material were purified and subjected to g.l.c./m.s. The spectra (not shown) for the putative uracil peak and authentic uracil were similar. The mass spectrum of the bistrimethylsilyl derivative of the putative uracil peak showed peaks as follows: *m/z* 256 [M^+ (the molecular ion)] (40%), 255 (M^+-H) (35%), 113 $[(CH_3)_2SiOCNCH]^+$ (35%), and 99 $[(CH_3)_2SiOCCH]^+$ (90% relative to the base peak at *m/z* 241, as M^+-CH_3) as well as the characteristic trimethylsilyl fragments at *m/z* 73 and 147 [18]. The bistrimethylsilyl derivative of authentic uracil showed similar peaks at *m/z* 256 (45%), 255 (35%), 147 (65%), 113 (40%) and 73 (80% relative to the base peak at *m/z* 99).

The effect of 15.6 nM-noradrenaline (steady state for pressure and O_2 corresponding to position C, Fig. 3) on the net release of the purine and pyrimidine metabolites is shown in Table 2. Uracil and uric acid were consistently and significantly increased. The increase in xanthine was also significant as compared with the basal state (Table

Table 2. Effect of noradrenaline, vasopressin, angiotensin II and sciatic-nerve stimulation on the release of uracil- and adenine-derived metabolites from the perfused rat hindlimb

Hindlimbs were perfused as described in the Experimental section and allowed to equilibrate to basal conditions (usually 40 min) before commencing the experiment. The agonists were then introduced while venous pO_2 and arterial pressure were continuously recorded. Separate hindlimbs were used for each agonist. Sciatic-nerve stimulation was applied to each hindlimb after removal of the agonist and when basal conditions had returned. Perfusate samples were collected at intervals of 5 min. After deproteinization with $HClO_4$ and neutralization, the supernatants were analysed by reverse-phase h.p.l.c. Means ± S.E.M. of steady-state values are shown. Significance of difference compared with 'None' was determined by Student's unpaired *t* test: *P* values are given (n.s., not significant).

Addition	*n*	Pressure (mmHg)	O_2 uptake (μmol/h per g)	Metabolite concn. (μM)							
				Uracil	Uric acid	Uridine	Hypoxanthine	Xanthine	Inosine	Guanosine	Adenosine
None	11	29±2	6.4±0.2	1.43±0.11	3.48±0.29	0.40±0.07	0.26±0.05	0.36±0.05	0.13±0.03	0.12±0.05	0.07±0.06
15.6 nM-Noradrenaline	5	40±2	9.3±0.3	2.63±0.28	5.77±0.62	0.36±0.08	0.49±0.11	0.8±0.13	0.25±0.06	0.15±0.03	0
		P < 0.01	*P* < 0.001	*P* < 0.001	*P* < 0.01	n.s.	n.s.	*P* < 0.01	n.s.	n.s.	n.s.
0.5 nM-Vasopressin	3	66±11	10.8±0.6	3.83±0.13	7.91±0.96	0.56±0.08	0.52±0.11	0.98±0.12	0.32±0.05	0.21±0.04	0
		P < 0.01	*P* < 0.01	*P* < 0.001	*P* < 0.001	n.s.	n.s.	*P* < 0.001	*P* < 0.05	n.s.	n.s.
5 nM-Angiotensin	3	54±3	10.3±0.7	7.08±1.27	19.9±4.48	2.56±0.57	0.35±0.03	0.55±0.04	0.24±0.06	0	0
		P < 0.001	*P* < 0.001	*P* < 0.001	*P* < 0.001	n.s.	*P* < 0.001	n.s.	n.s.	n.s.	n.s.
Sciatic-nerve stimulation	7	29±1	13.0±1.1	1.33±0.19	3.40±0.47	0.49±0.12	0.48±0.05	0.48±0.08	0.87±0.12	0.05±0.02	0
		n.s.	*P* < 0.001	n.s.	n.s.	n.s.	*P* < 0.05	n.s.	*P* < 0.001	n.s.	n.s.

2). Hypoxanthine and inosine tended to increase; however, these changes were not significant (Table 2). There were two unidentified minor peaks at 16.1 and 23.8 min which were unchanged. Adenosine could not be detected.

The effect of 5 min of intermittent sciatic-nerve stimulation (see the Experimental section) on the net release of pyrimidine and purine metabolites by the hindlimb is also shown in Table 2. The principal muscles contracting under these conditions were the gastocnemius–plantaris–soleus group. Tension measurements (results not shown) indicated that the initial load developed was approx. 900 g and that this decreased over the 5 min period to approx. 50 g unless the perfusion flow rate was increased. This might imply that energy demand exceeded supply during the final stages of this period of skeletal-muscle contraction. Perfusate samples were taken at position D (Fig. 3), and analysis showed that inosine was markedly increased (Table 2). Hypoxanthine was also significantly increased, but trends by uracil, uric acid, and xanthine were not significant. Two minor new peaks appeared at 20 and 29 min, but these were not identified. Adenosine could not be detected during skeletal-muscle contraction.

In addition to summarizing the data from experiments involving noradrenaline and sciatic-nerve stimulation, Table 2 shows data from experiments involving the effects of two vasoconstrictors, vasopressin (0.5 nM) and angiotensin II (5 nM). At the concentrations chosen, the effects of these agents tended to be greater than those of noradrenaline in terms of pressure development and O_2 uptake, and they produced a markedly greater release of uric acid and uracil than did the catecholamine. Angiotensin (5 nM) led to a 5-fold increase in uracil release and a 6-fold increase in both uric acid and uridine release.

Fig. 2 shows the dose–response curves for noradrenaline-mediated increases in uracil, uric acid and inosine release as well as O_2 uptake and perfusion pressure by the isolated perfused rat hindlimb. Apparent half-maximal stimulation of uracil and uric acid production occurred at 12 nM-noradrenaline; this was greater than for O_2 uptake and pressure development (9 nM-noradrenaline). Inosine release did not increase significantly until the concentration of noradrenaline exceeded 30 nM.

Fig. 3 shows the time-course relationships for uric acid, uracil and inosine release in relation to changes in O_2 uptake and pressure development after perfusion with 15.6 nM-noradrenaline as well as after sciatic-nerve stimulation. Uric acid and uracil release accompanied the increase in O_2 uptake and pressure development. Maximum release was reached at approx. 7 min after noradrenaline addition, and this preceded peak values for O_2 uptake and pressure by 5 min. In addition, maximum uric acid and uracil release was sustained during the noradrenaline infusion and returned to basal levels when the catecholamine was withdrawn (Fig. 3). Sciatic-nerve stimulation resulted in small transient increases in pressure and in uric acid and uracil release. A

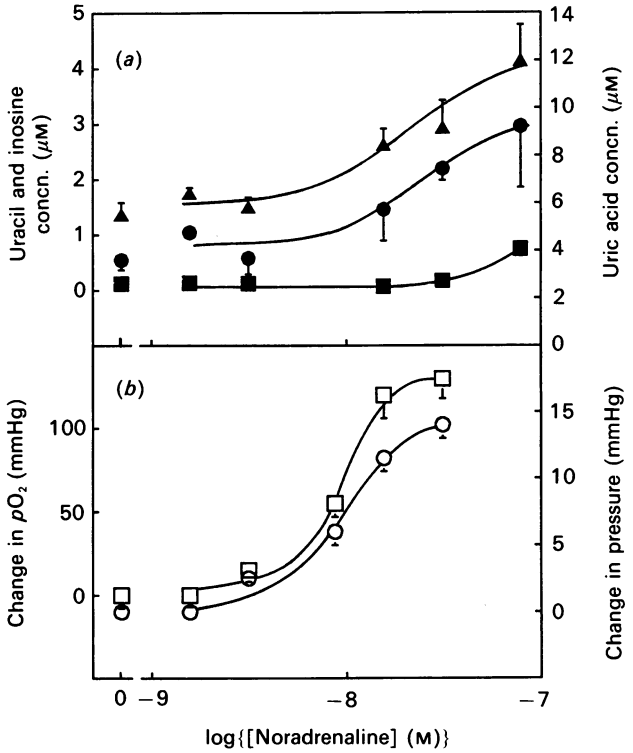

Fig. 2. Dose–response curves for noradrenaline-mediated increases in the release of uracil, uric acid and inosine by the perfused rat hindlimb

Hindlimbs were perfused as described in the Experimental section, and noradrenaline was introduced with a stepwise increment in dose from 1.5 to 78 nM. Perfusate samples were collected and analysed for uracil (▲), uric acid (●) and inosine (■) (*a*). Changes in venous pO_2 (□) as well as pressure (○) were also monitored (*b*). Steady-state values are shown as means ± S.E.M. for a minimum of three perfusions. When not visible, the error bar is within the symbol.

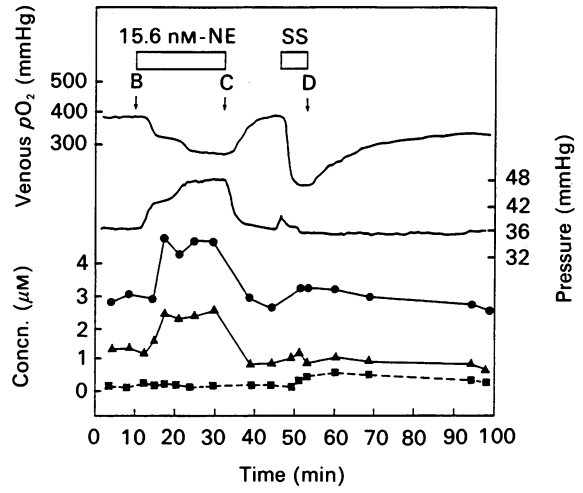

Fig. 3. Time course for the effect of noradrenaline and sciatic-nerve stimulation on venous pO_2, pressure and the perfusate concentrations of uric acid, uracil and inosine

Hindlimbs were perfused as described in the Experimental section, and 15.6 nM-noradrenaline (NE) was introduced for the 22 min period as shown. Details for sciatic-nerve stimulation (SS) were as given in the text. Venous pO_2 and arterial perfusion pressure were monitored continuously. Perfusate samples were collected and analysed for uric acid (●), uracil (▲) and inosine (■). The results are representative of five perfusions; mean steady-state values at the arrows appear in Table 2. A representative h.p.l.c. tracing for the sample taken at B appears in Fig. 1.

large increase in O_2 uptake was followed by a sustained release of inosine. Usually the maximum release of inosine occurred 5–10 min after the maximum increase in O_2 uptake.

DISCUSSION

The release of uric acid, uracil and uridine in close association with vasoconstriction, and not during skeletal-muscle contraction, implies that events during constriction of vascular muscle are central to the release. Constriction of the hindlimb vasculature by agonists such as noradrenaline [19,20,15,14], vasopressin and angiotensin [14] has been reported to be associated with an increase in hindlimb O_2 uptake. Possible explanations for the increase in O_2 uptake [14] are relevant to a consideration of the possible origins of uric acid, uracil and uridine, and include: (i) vasoconstricting agents acting at specific sites on resistance arterioles to increase pressure, resulting in access of perfusion medium to regions of the hindlimb that were previously underperfused; (ii) receptors for the vasoconstriction, in addition to those on the vasculature, are present on skeletal muscle and act directly to increase skeletal-muscle fibre O_2 uptake; and (iii) O_2 is consumed by the vascular smooth muscle as it constricts to increase and hold perfusion pressure [14].

Each of these three possibilities has implications for phosphagen turnover and the appearance of breakdown products. With regard to the first, heterogeneous perfusion has been noted in the auto-perfused teniussimus muscle of the rabbit when both pressure and flow were decreased [21]. In the present study perfusion rates were high. If a large proportion of the hindlimb was underperfused before the addition of the vasoconstricting agents, a release of tissue indicators of hypoxia/ischaemia such as adenosine, inosine and hypoxanthine would be expected. Instead, a marked increase in release of uric acid, uracil and uridine was noted (Table 2).

Vasoconstrictor-mediated increase in O_2 uptake via skeletal-muscle receptors would presuppose that the pressure increase is unrelated to the increase in O_2 uptake. In addition, this explanation would require a biochemical explanation for the increased O_2 uptake, such as mitochondrial uncoupling, ATPase activity or substrate cycling. Accelerated ATP hydrolysis leading to AMP formation, and then to IMP, inosine, hypoxanthine, xanthine and uric acid, could then occur. However, there is no obvious explanation to account for the co-release of uracil and uridine with uric acid during vasoconstriction.

The third possibility focuses on the vascular tissue, where we have proposed that O_2 is consumed by the vascular tissue as it constricts to increase and hold perfusion pressure [14]. If this is so, vascular muscle may show properties similar to skeletal [3] and cardiac [8] muscle, where intense work performed (e.g. during constriction) leads to an imbalance between energy demand and supply with the release of adenosine. There is already one study [22] where intravenous injection of adrenaline to adult rabbits was found to decrease ATP and ADP and to increase P_i of the aortic wall without formation of AMP. The lack of appearance of adenosine in the effluent may be explained by uptake into endothelial cells [4], where there are high activities of adenosine deaminase [23] and xanthine oxidase [24], leading to formation of uric acid.

Mechanical work by the vascular smooth muscle during intense constriction may also explain the release of uracil and uridine. Vascular smooth muscle differs significantly from skeletal muscle when the levels of purine and pyrimidine nucleotides are compared. Literature values for phosphagen contents of vascular tissue have been reviewed by Paul [25]. The average value for ATP can be calculated from those data and is 1.0 ± 0.29 μmol/g wet wt. (mean ± S.E.M. for 11 reports). Three of the 11 groups cited [25] reported concentrations of UTP + GTP, or unspecified organic phosphate, exceeding 0.3 μmol/g wet wt. [26–28]. One group indicated that GTP + UTP in bovine carotid artery was 1.21 μmol/g wet wt., thus approximating to half the concentration of ATP in this tissue of 2.68 μmol/g wet wt. [28]. In rat abdominal/thoracic aorta our own measurements of ATP and UTP were 0.70 ± 0.18 ($n = 3$) and 0.13 ± 0.01 ($n = 3$) μmol/g wet wt. respectively, giving a ratio of ATP/UTP of 5.4. For comparison, ATP and UTP contents of rat diaphragm are 3.98 and 0.062 μmol/g wet wt., with a ratio of ATP/UTP of 64 [29]. The absolute values for aorta have not been corrected for the actual smooth-muscle content, as opposed to the large amount of connective tissue in this elastic artery. In addition, the values for diaphragm including the ATP/UTP ratio do not take into account the inherent vascular smooth-muscle content. Thus the ratio of ATP/UTP for vascular smooth muscle versus striated muscle may differ by even more than the factor of 12 indicated above.

Data of Daemers-Lambert [28] suggest that UTP, like ATP, is involved in the energetics of smooth-muscle contraction. The concentration of all three phosphagens (ATP, UTP + GTP) decreased in parallel when iodoacetic acid-poisoned carotid-artery fragments were induced to contract with KCl [28]. However, a specific role of UTP in vascular tissue is not obvious. Biochemical texts (e.g. ref. [30]) show that UTP, apart from being a precursor for RNA synthesis, is predominantly involved in monosaccharide transfer reactions, e.g. glycogen synthase, UTP–α-D-galactose-1-phosphate uridylyltransferase. Thus further studies will be required to determine the precise metabolic origin of the uracil and uridine co-released with uric acid during vasoconstriction of the rat hindlimb. In addition, the involvement of pyrimidines in the regulation of cell functions [31] may need to be considered.

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