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Mechanisms in rabbit aorta for hyperglycaemia-induced alterations in angiotensin II and norepinephrine effects

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Summary. The (Na^+, K^+)-ATPase activity operative in rabbit aortic intima-media incubated with normal plasma levels of glucose and *myo*-inositol (70 µmol/l) is decreased when the glucose content of the medium is raised from 5 to 10 mmol/l or higher; this effect is prevented by aldose reductase inhibitors and by raising the *myo*-inositol content of the medium to 500 µmol/l. The decrease in (Na^+, K^+)-ATPase activity results from the loss of a component normally regulated (stimulated) by endogenously released adenosine through a receptor that stimulates phosphatidylinositol turnover in a discrete pool. The replenishment of this phosphatidylinositol pool selectively requires *myo*-inositol transport and is inhibited when increased polyol pathway activity impairs *myo*-inositol transport at a normal plasma level. Adenosine is a vasodilator, some endothelium-released vasodilators modulate the responses to vasoconstrictors by stimulating an increase in (Na^+, K^+)-ATPase activity in vascular smooth muscle. Whether adenosine mediates this effect in angiotensin II or norepinephrine-stimulated aorta was examined. Angiotensin II (100 nmol/l) and norepinephrine (1 µmol/l) evoked marked increases in (Na^+, K^+)-ATPase activity in aortic intima-media incubated with 5 mmol/l glucose and 70 µmol/l *myo*-inositol, which were inhibited when adenosine deaminase was added or the medium *myo*-inositol omitted to inhibit *myo*-inositol transport. Raising the medium glucose to 30 mmol/l inhibited the angiotensin II and norepinephrine-evoked increases in (Na^+, K^+)-ATPase activity, and this was prevented when tolrestat (10 µmol/l) was added or the *myo*-inositol content of the medium was raised from 70 to 500 µmol/l. Hyperglycaemia causes decreased (Na^+, K^+)-ATPase activity prevented by aldose reductase inhibitors and by raising plasma *myo*-inositol by a mechanism which inhibits an adenosine-(Na^+, K^+)-ATPase regulatory system, which modulates the responses to angiotensin II and norepinephrine in some blood vessels.

Key words: Aorta, (Na^+, K^+)-ATPase, adenosine, angiotensin II, norepinephrine, hyperglycaemia, vasodilation, vasoconstriction.

The vasopressor responses to i.v. angiotensin II and norepinephrine are increased in diabetic states, even when vascular complications and hyperinsulinaemia are absent, and the vasoconstrictive responses to these agonists are increased in specific arteries and arterioles in streptozotocin diabetic rats and alloxan diabetic rabbits [1–6]. Because there is no known mechanism by which hyperglycaemia could cause increased vasoconstrictive responses to angiotensin II or norepinephrine, it has received scant consideration as a possible causal factor. Vasoconstriction in arteries and resistance arterioles is normally modulated by endothelium-released vasodilators, and impaired modulation is a cause for increased vasoconstrictive responses [7]. The endothelium-derived vasodilators include a hyperpolarizing factor (or factors) of unknown structure that acts by stimulating increased electrogenic (Na^+, K^+)-ATPase activity in vascular smooth muscle [7, 8].

Hyperglycaemia decreases (Na^+, K^+)-ATPase activity and induces functional alterations in specific tissues by a mechanism whose identifying characteristic is that its effects are prevented in diabetic animals both by aldose reductase inhibitors and by raising their otherwise normal plasma *myo*-inositol (MI) levels by approximately sevenfold [9, 10]. It was recently found that this mechanism can be activated and studied in vitro in resting normal rabbit aortic intima-media (AIM) and that it inhibits a previously unknown adenosine (Na^+, K^+)-ATPase regulatory system [11–13]. Adenosine is an autocoid that is released by most tissues in a manner that varies with their activity [14]; it is released by endothelium and is a potent vasodilator in most arteries and resistance arterioles [15, 16]. Adenosine-induced vasodilation has been ascribed to A_2 receptors that stimulate adenylate cyclase in vascular smooth muscle, but current evidence suggests that this is

not the sole or primary vasodilatory mechanism of adenosine and the existence of some other mechanism(s) has been postulated [15]. In resting AIM incubated with normal plasma levels of glucose and MI, endogenously released adenosine regulates (stimulates) a distinct component of (Na^+, K^+)-ATPase activity through a novel type of A_2 receptor that stimulates rapid basal phosphatidylinositol (PI) turnover, apparently phospholipase C hydrolysis, in a discrete PI pool [12]. This PI pool is maintained by a distinct fraction of de novo PI synthesis that selectively requires MI transport at the low normal plasma MI level to prevent its rapid inhibition and the loss of the adenosine effect on (Na^+, K^+)-ATPase activity [12]. In AIM provided with medium MI in a normal plasma level raising the glucose level of the medium from 5 to 10 mmol/l or to 30 mmol/l selectively inhibits the adenosine-stimulated component of normal resting (Na^+, K^+)-ATPase activity within 60 min. This inhibition is prevented by aldose reductase inhibitors and by raising the MI concentration of the medium seven-fold; it results from increased polyol pathway activity that impairs MI transport at a normal plasma level and inhibits the replenishment of the adenosine-sensitive PI pool [11, 12].

We examined the possibility that when AIM is stimulated by angiotensin II or norepinephrine the hyperglycaemia-inhibitable, adenosine-(Na^+, K^+)-ATPase regulatory system normally stimulates an increase in vascular smooth muscle (Na^+, K^+)-ATPase activity to modulate the contractile response.

Materials and methods

Male, white New Zealand rabbits (2.0–2.5 kg) were fasted overnight, sedated with diazepam (2 mg/kg i. m.), and 90 min later anaesthetized with sodium pentobarbital (30 mg/kg i. v.) and decapitated. The descending aorta was rapidly excised and four tubular segments of AIM, each weighing approximately 30 mg, were prepared by a method that produces AIM that is free of adventitia and retains an intact endothelium with a normal electron microscopic appearance [17, 18]. All the media to which the aorta and AIM were exposed contained 9% clinical grade dextran (average mol. wt. 70,000) to provide the oncotic pressure that is required to preserve the normal ultrastructure and normal pattern of energy metabolism of AIM [17, 18]. The aorta was dissected in Krebs-Henseleit bicarbonate buffer [19] (which contains normal plasma concentrations of Na^+ and K^+), pH 7.4 at 37 °C, that contained normal rabbit plasma levels of glucose (5 mmol/l) and MI (70 μmol/l) and was continuously gassed with 5% CO_2/95% O_2.

For each experiment the four AIM segments from a single aorta were used to provide two paired samples, each comprised of duplicate AIM segments, in which (Na^+, K^+)-ATPase-mediated $^{86}Rb^+/K^+$ uptake was compared in similar medium in the absence and presence of angiotensin II (100 nmol/l) or in the absence and presence of norepinephrine (1 μmol/l). The method for determining (Na^+, K^+)-ATPase-mediated $^{86}Rb^+/K^+$ uptake in AIM was previously reported in detail [11, 12]. Briefly, the individual freshly prepared AIM segments were equilibrated for 30 min at 37 °C in 3 ml of Krebs-Henseleit bicarbonate buffer continuously gassed with 5% CO_2/air that contained 9% dextran and specified concentrations of glucose and MI, in a 10 ml Erlenmeyer flask in a metabolic shaker set at 88 cycles per min. The individual AIM segments were then transferred to fresh medium of the same composition that contained an added tracer quantity of [^{3}H]sucrose. Ouabain (0.2 mmol/l) was added to one of the duplicate AIM segments in each paired sample, and the tissues pre-incubated for 10 min, as described for the equilibration. At the end of the pre-incubation a tracer quantity of [$^{86}Rb^+$]RbCl was added to each flask, the vasoconstrictor whose effect was to be examined was added to the two AIM segments in one of the paired samples, and the tissues incubated for 20 min. At the end of the incubation the individual AIM segments were rapidly removed, drained on filter paper, weighed, and transferred into 2 ml of Protosol in a glass liquid scintillation vial and digested for 16 h. The tissue digest was decobrized with H_2O_2, Econoflor 2 added to the vial, and the sample counted for $^{86}Rb^+$ and ^{3}H in a liquid scintillation spectrometer with an external standard. A sample of the incubation medium was also counted and the medium [K^+] determined. The $^{86}Rb^+$-counts recovered in the tissue digest were corrected for the $^{86}Rb^+$-counts trapped in the extracellular fluid in the tissue sample by means of the [^{3}H]sucrose-counts recovered in the tissue digest and the ratio of $^{86}Rb^+$-counts/[^{3}H]sucrose-counts in the incubation medium. The resulting estimate of intracellular $^{86}Rb^+$-counts was expressed as μmol $^{86}Rb^+/K^+$ uptake $\cdot g^{-1} \cdot 20\ min^{-1}$ by means of the ratio of the medium K^+ concentration to the medium $^{86}Rb^+$-counts per unit volume. The difference between the uptakes in the absence and presence of ouabain in the two AIM segments in each paired sample was used as an estimate of the (Na^+, K^+)-ATPase-mediated $^{86}Rb^+/K^+$ uptake.

The sources of the materials used were: dextran, ouabain, angiotensin II, norepinephrine bitartrate (Sigma Chemical Co., St. Louis, Mo. USA); *myo*-inositol (Pfhanstiehl Labs. Inc., Waukegan, Ill., USA); adenosine deaminase (Boehringer-Mannheim Biochemicals, Indianapolis, Ind., USA); Protosol, Econoflor 2, [$^{86}Rb^+$]RbCl, [^{3}H]sucrose (New England Nuclear, Boston, Mass., USA). Tolrestat was a gift from Wyeth-Ayerst Research (Princeton, N.J., USA).

Statistical analysis

The mean $\Delta \pm$ SEM between the paired samples incubated in the absence and presence of angiotensin II or norepinephrine in each group of experiments was analysed for significance by a paired Student's *t*-test. In specific instances noted in the text and tables the difference in the mean $\Delta \pm$ SEM induced by angiotensin II or norepinephrine in two groups of paired experiments was analysed for significance by an unpaired Student's *t*-test.

Results

Effects of angiotensin II (100 nmol/l)

In paired AIM samples equilibrated and incubated with normal rabbit plasma levels of glucose (5 mmol/l) and MI (70 μmol/l), added angiotensin II evoked a significant increase in (Na^+, K^+)-ATPase-mediated $^{86}Rb^+/K^+$ uptake (Table 1); the mean percent increase ± SEM was 96 ± 13%. When these experiments were repeated with adenosine deaminase (1 U/ml) added to the medium in both samples to degrade endogenously released adenosine the addition of angiotensin II had no significant effect on (Na^+, K^+)-ATPase activity (Table 1). In paired AIM samples that were equilibrated and incubated in medium containing 5 mmol/l glucose, but with the medium MI omitted to inhibit MI transport and the replenishment of the adenosine-sensitive PI pool, the addition of angiotensin II also had no significant effect on (Na^+, K^+)-ATPase activity (Table 1).

In paired AIM samples equilibrated and incubated in medium containing 30 mmol/l glucose and MI at a normal

Table 1. Effects of angiotensin II (100 nmol/l) on (Na^+, K^+)-ATPase activity in rabbit aortic intima-media (AIM)

Incubation medium	Effect on (Na^+, K^+)-ATPase-mediated $^{86}Rb^+/K^+$ uptake ($\mu mol \cdot g^{-1} \cdot 20\ min^{-1}$)			
Glucose (mmol/l)	*myo*-inositol (µmol/l)	Mean Δ ± SEM	*n*	*p*
5	70	+ 1.61 ± 0.15[a]	(15)	< 0.001
+ adenosine deaminase (1 U/ml)				
5	70	+ 0.43 ± 0.33	(10)	NS
5	None	+ 0.46 ± 0.23	(9)	NS
30	70	+ 0.21 ± 0.28	(16)	NS
30	500	+ 1.45 ± 0.22[a]	(7)	< 0.001
+ tolrestat (10 µmol/ml)				
30	70	+ 1.46 ± 0.22[a]	(10)	< 0.001

Values are the mean difference ± SEM in (Na^+, K^+)-ATPase-mediated $^{86}Rb^+/K^+$ uptake in paired AIM samples from the same aorta incubated in the absence and presence of angiotensin II (100 µmol/l) for 20 min. The paired AIM samples were equilibrated (30 min), pre-incubated (10 min), and incubated (20 min) in Krebs-Henseleit bicarbonate buffer, pH 7.4 at 37 °C, gas phase 5% CO_2/air that contained 9% dextran (average mol. wt. 70,000), and glucose and *myo*-inositol in the concentrations noted in the table. In specific experiments adenosine deaminase or tolrestat was added to the medium in both paired samples during the equilibration, pre-incubation, and incubation. *p* was determined by a paired Student's *t*-test analysis. [a] The magnitudes of these effects were not significantly different from each other when analysed by an unpaired Student's *t*-test

plasma level (70 µmol/l), added angiotensin II had no significant effect on (Na^+, K^+)-ATPase activity (Table 1). However, when these experiments were repeated with the medium MI level raised to 500 µmol/l the addition of angiotensin II evoked a significant increase in (Na^+, K^+)-ATPase-mediated $^{86}Rb^+/K^+$ uptake, and the magnitude of this effect was not significantly different from that observed in AIM incubated with normal plasma levels of glucose and MI (Table 1). Angiotensin II evoked a significant increase in (Na^+, K^+)-ATPase activity in AIM equilibrated and incubated in medium containing 30 mmol/l glucose and MI at a normal plasma level when an aldose reductase inhibitor, tolrestat (10 µmol/l), was added to both paired samples (Table 1); the magnitude of this effect was not significantly different from that observed in AIM incubated with normal plasma levels of glucose and MI (Table 1).

Effects of norepinephrine (1 µmol/l)

Norepinephrine evoked a significant increase in (Na^+, K^+)-ATPase activity in AIM equilibrated and incubated with normal plasma levels of glucose and MI (Table 2); the mean percent increase ± SEM was 96 ± 26%. When these experiments were repeated with adenosine deaminase (1 U/ml) added to the medium the addition of norepinephrine caused no significant change in (Na^+, K^+)-ATPase activity (Table 2). Norepinephrine also had no significant effect on (Na^+, K^+)-ATPase activity in AIM equilibrated and incubated with 5 mmol/l glucose when MI was omitted from the medium (Table 2).

Norepinephrine caused no significant change in (Na^+, K^+)-ATPase activity when added to AIM equilibrated and incubated with 30 mmol/l glucose and MI in a normal plasma level (Table 2). However, in AIM equilibrated and incubated with 30 mmol/l glucose the addition of norepinephrine did evoke significant increases in (Na^+, K^+)-ATPase activity when the MI content of the medium was raised to 500 µmol/l or when the MI content remained at 70 µmol/l and tolrestat (10 µmol/l) was added to the medium (Table 2); the magnitudes of these effects were not significantly different from those observed in AIM incubated with normal plasma levels of glucose and MI (Table 2).

Discussion

Hyperglycaemia decreases (Na^+, K^+)-ATPase activity and induces early functional alterations in specific organs and regions of the vascular and nervous system by a mechanism whose effects can be prevented in diabetic animals both by aldose reductase inhibitors and by raising normal plasma MI levels approximately seven-fold [9, 10, 20]; we term this the "common initiating mechanism" [9]. Recent in vitro studies of this mechanism in resting AIM demonstrated that it decreases (Na^+, K^+)-ATPase activity by inhibiting the effector arm of a novel adenosine-(Na^+, K^+)-ATPase-regulatory system by which endogenously released adenosine regulates (stimulates) and normally maintains a distinct component of resting (Na^+, K^+)-ATPase activity in AIM. The present study was concerned with determining whether one of the physiological functions of this regulatory system is to stimulate an increase in (Na^+, K^+)-ATPase activity in vascular smooth muscle when resting AIM is stimulated by angiotensin II or norepinephrine to modulate the contractile response.

When resting rabbit AIM with an intact endothelium is incubated with normal plasma levels of glucose (5 mmol/l) and MI (70 µmol/l) in Krebs-Henseleit bicarbonate buffer containing 9% dextran, it maintains a stable rate of (Na^+, K^+)-ATPase activity, (comprised of two distinct components), that is responsible for a major fraction of the energy utilization of the tissue [11–13]. Under these conditions the addition of angiotensin II (100 nmol/l) or of norepinephrine (1 µmol/l) in a concentration known to induce contraction in rabbit aorta [21] evoked a marked increase in (Na^+, K^+)-ATPase activity. The addition of angiotensin II or norepinephrine had no significant effect on

Table 2. Effects of norepinephrine (1 µmol/l) on (Na^+, K^+)-ATPase activity in rabbit aortic intima-media (AIM)

Incubation medium	Effects on (Na^+, K^+)-ATPase-mediated $^{86}Rb^+/K^+$ uptake (µmol·g^{-1}·20 min^{-1})			
Glucose (mmol/l)	*myo*-inositol (µmol/l)	Mean Δ ± SEM	*n*	*p*
5	70	+1.52 ± 0.30[a]	(10)	< 0.01
+ adenosine deaminase (1 U/ml)				
5	70	+0.38 ± 0.26	(9)	NS
5	None	+0.56 ± 0.24	(9)	NS
30	70	+0.13 ± 0.14	(8)	NS
30	500	+1.15 ± 0.14[a]	(8)	< 0.001
+ tolrestat (10 µmol/l)				
30	70	+1.10 ± 0.32[a]	(8)	< 0.05

Values are the mean difference ± SEM in (Na^+, K^+)-ATPase-mediated $^{86}Rb^+/K^+$ uptake in paired AIM samples incubated in the absence and presence of norepinephrine (1 µmol/l) for 20 min. The experiments were performed as described in the legend for Table 1. In the experiments in which adenosine deaminase or tolrestat was added to the medium, it was present in both paired AIM samples during the equilibration, pre-incubation, and incubation. *p* was determined by a paired Student's *t*-test analysis.

[a] The magnitudes of these effects were not significantly different from each other when analysed by an unpaired Student's *t*-test

(Na^+, K^+)-ATPase activity when adenosine deaminase (1 U/ml) was added to the medium to degrade endogenously released adenosine, which is a standard means of determining whether a biological effect is dependent upon endogenously released adenosine [22]. The addition of angiotensin II or norepinephrine also had no significant effect on (Na^+, K^+)-ATPase activity when the MI was omitted from the medium to inhibit MI uptake and selectively inhibit the replenishment of the adenosine-sensitive PI pool [12, 13]. Previous studies demonstrated that in AIM incubated with 5 mmol/l glucose the omission of MI from the medium at a normal plasma level or the addition of a competitive inhibitor of MI transport selectively inhibits the replenishment of the adenosine-sensitive PI pool and the adenosine-stimulated component of resting (Na^+, K^+)ATPase activity; under these conditions the ability of specific adenosine analogues to stimulate (Na^+, K^+)-ATPase activity in AIM is also inhibited [12, 13]. Although MI in a normal plasma level in the medium is required to demonstrate the effect of endogenously released adenosine on (Na^+, K^+)-ATPase activity in AIM, there is no requirement for MI in the medium to demonstrate angiotensin II or norepinephrine-induced contraction in rabbit aorta [23]. These observations indicate that angiotensin II or norepinephrine stimulation of resting AIM normally evokes a marked adenosine-stimulated increase in (Na^+, K^+)-ATPase activity that is mediated through the novel adenosine-(Na^+, K^+)-ATPase regulatory system. One can safely assume that the marked increases in total (Na^+, K^+)-ATPase activity of AIM that were observed primarily reflected adenosine-stimulated increases in vascular smooth muscle (Na^+, K^+)-ATPase activity, because the mass of vascular smooth muscle cells in AIM is much greater than that of endothelial cells.

Stimulation of increased electrogenic (Na^+, K^+)-ATPase activity in vascular smooth muscle is a vasodilatory mechanism, which modulates the component of vascular smooth muscle contraction that is dependent on membrane depolarization [7, 8, 24]. This is the distinctive vasodilatory mechanism of an endothelium-derived hyperpolarizing factor (or factors) of unknown structure, which plays a role in the modulation of vasoconstriction in arteries that is distinct from that of nitric oxide [7, 8]. Adenosine is a vasodilator in aorta and most other arteries and resistance arterioles outside of the kidney and placenta, and it has a direct vasodilatory effect in vascular smooth muscle [15]. Vasodilatory effects of adenosine cannot be attributed solely or primarily to A_2 receptors that stimulate adenylate cyclase in vascular smooth muscle and the existence of some other vasodilatory mechanism has been postulated [15]. The observation that adenosine is the endogenously released vasodilator that stimulates the increase in (Na^+, K^+)-ATPase activity in AIM that occurs in response to stimulation by angiotensin II or norepinephrine supports the inference that adenosine normally modulates the contractile responses to these vasoconstrictors in aorta, and presumably some other vessels, by stimulating increased electrogenic (Na^+, K^+)-ATPase activity in vascular smooth muscle.

In resting AIM provided with MI at a normal plasma level in the medium, raising the glucose level from 5 to 10 mmol/l or to 30 mmol/l during a 30-min equilibration and 30-min incubation selectively inhibits the adenosine-stimulated component of (Na^+, K^+)-ATPase activity; the ability of specific adenosine analogues (e.g. 2-chloroadenosine) to stimulate (Na^+, K^+)-ATPase activity is also lost [11, 12]. The effects of endogenously released adenosine and of 2-chloroadenosine on (Na^+, K^+)-ATPase activity are preserved in AIM exposed to 30 mmol/l glucose when the MI content of the medium is raised from 70 to 500 µmol/l or when an aldose reductase inhibitor (tolrestat 10 µmol/l) is added, because the replenishment of the adenosine-sensitive PI pool is not inhibited under these conditions [11, 12]. Angiotensin II and norepinephrine did not evoke significant changes in (Na^+, K^+)-ATPase activity in AIM equilibrated and incubated with 30 mmol/l glucose and MI at a normal plasma level. However, when an aldose reductase inhibitor (tolrestat 10 µmol/l) was added or when the MI content of the medium was raised to 500 µmol/l, angiotensin II and norepinephrine evoked significant increases in (Na^+, K^+)-ATPase activity, whose magnitudes were similar to those evoked in AIM when incubated with normal plasma levels of glucose and MI. These observations demonstrate that

activating the "common initiating mechanism" in AIM inhibits the adenosine-stimulated increases in (Na^+, K^+)-ATPase activity that normally play a role in the modulation of the responses to angiotensin II and norepinephrine. They indicate the existence of a mechanism by which hyperglycaemia could induce the increased vasoconstrictive responses to angiotensin II and norepinephrine that are observed in some arteries and resistance arterioles in diabetic states, including those in which hyperinsulinaemia is absent [1–6].

Hyperglycaemia is known to act through the "common initiating mechanism" to cause diverse early derangements in vascular regulation in diabetic states [9, 20, 25, 26]. Our observations provide a framework for efforts to understand the pathogenesis of these derangements, whose application requires attention to the regional differences in vascular regulation. They indicate that in blood vessels in which hyperglycaemia activates the "common initiating mechanism" its potential effects are not restricted to those resulting from a chronic reduction in (Na^+, K^+)-ATPase activity in the affected cells, but also include effects resulting from the loss of the acute autoregulatory effects that are normally mediated through the adenosine (Na^+, K^+)-ATPase regulatory system in that region of the vascular system. The latter could include altered responses to specific vasoactive hormones and neurotransmitters and derangements in the autoregulation of regional blood flow induced by the loss of one of the vasodilatory mechanisms of adenosine.

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