ATP and norepinephrine contributions to sympathetic vasoconstriction of tail artery are altered in streptozotocin-diabetic rats

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DIABETES is associated with a myriad of complications, including neuropathies and vasculopathies (4, 22, 27). Several studies have reported changes in sympathetic control of blood vessels in animal models of diabetes, although there has been wide variation in vascular responses to exogenous adrenergic agonists or nerve stimulation. Whatever the effects, it is often thought that autonomic neuropathy underlays these (4, 8, 16, 27). A common finding is that vessels from diabetic rats (7, 28–30) and rabbits (8, 26) have increased sensitivity to adrenergic agonists, although this observation is not universal (16, 19, 22, 25). Similarly, effects of diabetes on sympathetically evoked responses have varied from vasoconstriction being increased (26, 29, 30), remaining the same (8, 21, 25), or decreasing (16). In several studies a reduction in neuronal uptake of norepinephrine was thought to account for vasoconstrictor supersensitivity (8, 26). At present, no clear pattern of pathology for sympathetic regulation of vasculature in diabetes has emerged. In part, this inconsistency can be explained by the different animal models used, especially the duration of the diabetes relevant to the human pathology (10).

Few, if any, vascular sympathetic nerves rely solely on norepinephrine as a neurotransmitter (20). Norepinephrine is usually coreleased with other neurotransmitters, particularly ATP and neuropeptide Y (NPY) in rat tail and mesenteric arteries, each having a synergistic effect on each other (5, 22, 23). Furthermore, the role of each cotransmitter varies with nerve impulse patterns: norepinephrine contributes more to sustained trains of impulses, and ATP mediates responses to smaller numbers of impulses (5, 22, 23). Extraneuronal uptake of norepinephrine is negligible in this tissue (24). Despite this established observation, there has been little work on how the contribution from ATP is affected in pathologies such as diabetes.

The aim of the present study was to determine whether there is any change in contributions from both ATP and norepinephrine to sympathetically evoked neural responses and in response to exogenous norepinephrine and ATP in well-established (>3 mo) streptozotocin-induced diabetes. Some of this work has been published in abstract form (13).
Methods to record mechanical responses have been described in detail elsewhere (5) and are described here briefly. Animals were killed by cervical dislocation. Rings of artery (2–4 mm long) were taken from the proximal 5 cm of the tail from freshly killed rats and denuded of endothelium. Segments were mounted on stainless steel hooks within 4 ml tissue baths perfused (at 2 ml/min) with Krebs-Henseleit solution of the following composition (in mM): 118.4 NaCl, 4.75 KCl, 25 NaHCO3, 1.19 KH2PO4, 1.18 MgSO4, and 0.95 CaCl2. The normal glucose concentration was 5 mM (nondiabetic injected and control groups), and a unified high glucose concentration of 25 mM was used for vessels from the diabetic and high glucose control groups, a value used previously (10). Bath contents were kept at 37°C and continually bubbled with 5% CO2-95% O2 (pH of 7.4). Segments of vessels were suspended with fine metal hooks attached to cotton thread in the baths and attached to force transducers (Piodem, UF1, 25 g; Digitimer). Vessel responses were amplified (Neurolog NL108, Digitimer) and digitized using a lab interface (Micro 1401, C.E.D.) and data acquisition software (Spike 2, C.E.D.). A resting tension on vessel segments was set to 0.75 g and left to stabilize for 1 h. Field stimulation was delivered by two parallel platinum wires placed on either side of the vessel (5 mm separation) with a supra-maximal stimulus (6 V, 1 ms duration). All drug concentrations refer to final concentration in the tissue baths. Electrically evoked responses were completely abolished by tetrodotoxin (1 μM) or guanethidine (10 μM), confirming that responses were sympathetically evoked. Because no vasodilatation was observed in the presence of sympathetic blockade, the possibility of a sensory nerve contribution to the vessel responses was unlikely. Similarly, in pilot experiments (n = 3) preconstricted arteries (phenylephrine, 10–6 M) failed to dilate on addition of the sensory nerve stimulant capsaicin (10–5 M), although acetylcholine (10–6 M) caused significant (>50%) dilatation.

Protocols. The protocol used for the nerve stimulation/contractions studies is shown in Fig. 1. KCl (60 nM) was added to the bathing solution under stop-flow conditions to verify the contractile function of the rings, followed by washout (15 min). Usually cumulative concentration-effect curves for ATP (0.001–100 μM) or norepinephrine (0.001–100 μM) were then produced. Experiments were continued after washout and stabilization of baseline (>45 min) after the peak response to the final addition of norepinephrine. The preparation was then stimulated by using trains containing 1, 2, 4, 8, 10, 12, 16, 20, 30, and 100 impulses (supramaximal stimulation of 6 V, 1-ms duration, 20 Hz; Fig. 1). Trains were separated by intervals of 60 s (1–10 pulses), 90 s (12–20 pulses), or 120 s (20–100 pulses) to allow time for recovery between successive trains. For the sake of clarity, data for 10-, 16-, and 30-impulse responses are not shown in Figs. 3 and 4. The stimulus regime was then repeated in the presence of either 1) P2 purinoceptor antagonist suramin (100 μM; Fig. 1A), 2) α-adrenoceptor antagonist phentolamine (2 μM; Fig. 1B), or 3) P2X-antagonist NF279 (1 μM; Fig. 1). An incubation time of 10 min was used for phentolamine and 45 min for both suramin and NF279. These doses of phentolamine and suramin were shown previously (5) to completely inhibit the contractile responses to exogenous norepinephrine (1 μM) and ATP (100 μM). In the absence of previous data on the efficacy of NF-279 in the rat tail artery, we chose a concentration that consistently abolished the response to ATP (100 μM) in pilot studies. Figure 1 also illustrates a test for antagonist efficacy by its ability to block a single concentration (1 μM norepinephrine or 100 μM ATP) as shown. At the end of the experiment, KCl was tested again to verify sustained contractility: if <90% of the first application, the data were discarded.

Neurally evoked responses before and after α-adrenoceptor or P2 purinoceptor antagonists were examined in separate vascular rings. Phentolamine is an antagonist at both α1- and α2-receptor subtypes and was selected because both receptors are known to contribute postjunctionally in rat tail artery (1).

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Fig. 1. A and B: contractions of rings of rat tail artery to KCl, nerve stimulation (Stim), ATP, and norepinephrine. Typical protocol involved recording responses to KCl (60 nM) before control stimulation with 1–100 impulses at 20 Hz. Efficacy of antagonists suramin (Sur, 100 μM, A), NF-279 (1 μM, not shown), or phentolamine (Phent, 2 μM, B) were tested by abolition of responses to agonists ATP (1 μM) or norepinephrine (NE, 1 μM). This was followed by the same stimulation sequence in the presence of agonist. A final addition of KCl to check for presence of a response comparable to the initial test was then carried out. C: responses to stimulus trains of 5 impulses at 20 Hz were potentiated after 15 min incubation with desipramine (1 μM) to the same extent in all experimental groups (control tissue shown). Figure shows consecutive data recorded from three separate rats in A–C.
Separate experiments were conducted to exclude possible changes in norepinephrine uptake. Responses to five impulses at 20 Hz before and after inhibition of neuronal uptake of norepinephrine by 10 min incubation with desipramine (1 μM) were studied. Peak responses to three trains were averaged and compared before and after 15 min incubation with desipramine. In addition, concentration-response curves for norepinephrine were conducted in the presence of desipramine.

Histochemical study of sympathetic innervation density. Proximal tail arteries were dissected, and 5-mm transverse segments were cut open longitudinally and pinned out to their original in vivo length under phosphate-buffered saline (PBS in mM: 137 NaCl, 10.1 Na₂HPO₄, 1.84 KH₂PO₄, and 2.68 KCl; pH 7.4). These were fixed in 4% paraformaldehyde in PBS for 20 min at room temperature and washed for 2 h in PBS blocked with 1% bovine serum albumin (Sigma) in PBS and Triton X-100 (0.25%). Two series of experiments were undertaken: one using the neuronal marker polyclonal rabbit anti-protein gene product 9.5 antibody (PGP 9.5) antibody (at 1:200) and the other with anti-tyrosine hydroxylase antibody (at 1:200), which is specific for catecholamine-containing axons. PGP-9.5 antibody (Santa Cruz) was applied for 1 h at room temperature. Finally, secondary antibody Alexa Fluor 488 anti-rabbit antibody (Molecular Probes adapted from Ref. 12). After an additional wash in PBS, sections were incubated with tissue for 24 h at 4°C (both protocols adapted from Ref. 12). An additional wash in PBS, secondary antibody Alexa Fluor 488 anti-rabbit antibody (Molecular Probes) at 1:100 was applied for 1 h at room temperature. Finally, arteries were washed again in PBS, mounted in Vectashield (Vector Laboratories), and viewed on a Leitz Diaplan microscope equipped with confocal imaging (MRC600; Bio-Rad). For tyrosine hydroxylase antibody (Santa Cruz), tissue was treated similarly, except that incubation with primary antibody was for 16 h at 4°C, and the secondary antibody Alexa Fluor 568 (Molecular Probes) was applied for 1 h at room temperature. Images were taken at ×63 (image size: 768 × 512 pixels, 211 × 141 μm). Two layers 1-μm apart were projected together by using software (Confocal Assistant 4.02). Four different fields were imaged for each vessel. The average nerve intersection density (ID, Ref. 9) was measured as the number of times fluorescent structures crossed four lines of a superimposed square grid of 110 μm from the four images.

Data analysis. Results are expressed as means ± SE. Responses evoked by electrical stimulation during control conditions were expressed as grams. Whenever possible, statistical comparisons were made between tensions and assessed using two-way ANOVA followed by Student-Newman-Keuls post hoc test when appropriate. Responses evoked by electrical stimulation in the presence of an antagonist are expressed graphically (Fig. 3) as a percentage of the previous control. However, the majority of statistical comparisons were still performed on absolute data. Some comparisons made between normalized data were assessed with a paired Wilcoxon signed-rank test or Mann-Whitney test (stated). EC₅₀ data and intersection densities were compared by using paired and unpaired Student’s t-tests or one-way ANOVA, as appropriate (stated). Statistical significance was assumed at P < 0.05.

Drugs used. The following drugs were used: norepinephrine tartrate (Abbott, Queensborough, UK), desipramine hydrochloride, phentolamine hydrochloride, adenosine 5'-triphosphate disodium salt, suramin sodium (Sigma-Aldrich, Dublin, Ireland), and NF-279 (Tocris). All drugs were added to the baths under stop-flow conditions while being constantly bubbled with CO₂–O₂ mixture so that the final concentration of drug in the bath was as indicated.

RESULTS

Effects of KCl. Rings of tail artery were tested with KCl (60 mM) to assess contractility of vascular smooth muscle, independent from neural stimulation. The contractile effects were similar for arteries from the four test groups. Thus peak contractions were the following: diabetic, 0.89 ± 0.06 g, n = 35; nondiabetic injected, 0.88 ± 0.07 g, n = 25; control, 0.87 ± 0.05 g, n = 24; high glucose control 0.86 ± 0.07 g, n = 14.

Effects of electrical stimulation. Electrical stimulation evoked smooth contractures that were greater as impulse numbers were increased (Fig. 1). Figure 2A shows that stimulus-induced contractions were similar in vessels from the diabetic group and the three nondiabetic groups, irrespective of the pulse train used.

Responses to electrical stimulation in the presence of ATP antagonists. Responses to electrical stimulation in the presence of suramin in all nondiabetic groups were markedly reduced (Figs. 2B and 3A), and this reduction was more marked at lower impulse numbers (50–75% reduction in response to 1 impulse) compared with that at higher impulse numbers (15–25% reduction in response to 100 impulses; P < 0.05, 1 vs. 100 impulses nondiabetic injected tissue, Wilcoxon signed-rank), which accords with previous work (5, 23). The three nondiabetic groups yielded similar inhibition profiles, whereas the diabetic group profile of inhibition was clearly different, being uniform across the range of impulse numbers (difference between groups, P < 0.001, two-way ANOVA), independent of the length of pulse train used (65–68% reduction; Fig. 3A). When responses to individual impulse numbers in the diabetic group were compared with those of nondiabetic injected groups, reductions in the presence of suramin were different with impulses ≥8 (P < 0.01–0.001, two-way ANOVA). In the majority of experiments, the presence of suramin caused an obvious decrease in neurally evoked responses. Yet, as reported previously (2, 3, 15), on occasions responses were...
potentiated in the presence of suramin, although this did not appear to be related to the group stimulated (number of experiment with potentiated responses: nondiabetic injected, 2/14; noninjected normal, 0/15; high glucose, 2/12; diabetic, 1/31).

The P2X antagonist NF-279 (1 μM) depressed the evoked contractions also in manner dependent on stimulus parameters (Fig. 3) for control and nondiabetic injected groups (82–86% reduction in response to 1 impulse; 60–75% reduction in response to 100 impulses; \( P < 0.05 \), 1 vs. 100 impulses nondiabetic injected tissue, Wilcoxon signed-rank). The effect was consistently greater than with suramin (Figs. 2B and 3B).

Again, responses of the diabetic group vessels showed a greater susceptibility to NF-279 (84–89% depression: \( n = 11–25; \ P < 0.001 \), 1 vs. 100 impulses Wilcoxon signed-rank). Although diabetic group artery responses were inhibited to a slightly greater extent with all impulse numbers, these differences were not significant.

Responses to electrical stimulation in the presence of nor-epinephrine antagonist. Phentolamine (Figs. 2B and 3C) had an even more marked depressant effect. As with our previous studies (5), this reduction was slightly more pronounced at higher impulse numbers (88–92% in response to 100 impulses) compared with that at lower impulse numbers (73–86% reduction in response to one impulse; \( P < 0.05 \), 1 vs. 100 impulses nondiabetic injected tissue, Wilcoxon signed-rank). Mean reductions in responses from diabetic tissue were not significantly different between high and low impulse numbers.

Contraction with exogenous ATP. Bath-applied ATP (0.1–1,000 μM) produced a concentration-dependent contracture (Fig. 4A). Diabetic group vessels were more sensitive as indicated by the leftward shift (\( P < 0.01 \), difference between groups, one-way ANOVA). Because there was no plateau (see also Ref. 14), \( EC_{50} \) values could not be determined; therefore, comparisons were made at the highest ATP concentration. Here there was an ~26% increase for the diabetic group arteries compared with the arteries of all three other groups (\( P < 0.001 \), one-way ANOVA), whereas there were no differences between these other groups.

Contraction with exogenous norepinephrine. Norepinephrine (0.0001–100 μM) produced a concentration-dependent contracture, which did show plateaus (Fig. 4B) that were not different between groups. There was an increased sensitivity in arteries from both diabetic animals and from high glucose control animals (\( P < 0.05 \); Fig. 4B). Thus \( EC_{50} \) values were the following: diabetic group, 0.39 ± 0.10 μM (\( n = 24 \)); nondiabetic injected group, 1.81 ± 0.28 μM (\( n = 22; P < 0.01 \), one-way ANOVA); control, 1.36 ± 0.48 μM (\( n = 24 \)); and high glucose control group 0.48 ± 0.15 μM (\( n = 12; P < 0.05 \)).

Effects of blocking of norepinephrine uptake. The increased norepinephrine potency in the diabetic and high glucose control groups may reflect an attenuated norepinephrine uptake/reuptake. If so, inhibition of uptake would show a potentiation of nondiabetic group responses but not diabetic. In desipramine (1 μM, Fig. 1C), no such selectivity was observed, and all three tissues studied showed similar effects (diabetic increased by 79 ± 10%, \( n = 15; P < 0.001 \), nondiabetic by 73 ± 10%, \( n = 13; P < 0.0001 \); control tissue: by 103 ± 14%,
ATP and norepinephrine as cotransmitters in diabetes

**DISCUSSION**

We have provided evidence that in the tail artery of diabetic rats, there is a greater contribution from ATP to both neurogenic contraction and a more potent action of exogenously applied ATP. This appears to be the first report of this nature. We also confirm previous reports of an increased contribution of norepinephrine in arteries of diabetic rats that cannot be ascribed to defective reuptake. Despite increased sensitivities to ATP and norepinephrine, the magnitudes of neurogenic responses are no different in the diabetic group. We also show that diabetes had no effect on the density of innervation of tail artery.

Responses evoked by potassium chloride and by electrical stimulation. KCl, which causes depolarization primarily through voltage-dependent calcium channels, confirmed there were no differences in smooth muscle contractile function between diabetic and nondiabetic groups, in agreement with previous studies (7, 8, 19, 22). Thus any changes in purinergic and noradrenergic responses are likely to reflect transmitter release or receptor coupling.

The electrical responses were similar whether or not from diabetics and independent of stimulus numbers. This accords with results from the mesenteric bed 8 wk after streptozotocin injection (21, 21) and rabbit carotid artery 6 wk after alloxone (8). But in the rat mesenteric bed after 12 wk of diabetes (as in our study), sympathetically evoked responses were reduced (19, 31). Such differences might arise from time dependency or severity of diabetes and variation in its influence between blood vessels.

Increased sensitivity to exogenous agonists. Arteries from diabetic rats were more sensitive to exogenous ATP compared with nondiabetic animals, but because no supramaximal concentration was achievable, a distinction between receptor density and receptor binding/coupling could not be made. The only related study used perfused mesenteric bed of diabetic rats, but the potency of ATP or norepinephrine (22) was unaffected. Perhaps in the in vivo testing, an intact endothelium and sensory innervation might account for the difference.

Bath-applied norepinephrine produced the same supramaximal effect in diabetic and nondiabetic arteries, suggesting that receptor density was unaffected. Voltage-dependent calcium channels have been thought to explain enhanced norepinephrine action rat tail (28) and mesenteric artery (22). This seems unlikely because KC1 response was unaffected in retinal arterioles (10) or tail artery (28). More efficacious cell signaling is more likely because increased α1-adrenoceptor sensitivity mirrors an improved G protein or phospholipase-C coupling in the tail artery from diabetic rats (30).

Contributions of norepinephrine and ATP to neurogenic contractions. Suramin action in normal arteries implies a greater role for ATP in neurogenic responses at low impulse numbers, as shown previously (1, 5, 23), but NF-279 caused greater inhibition of all neurogenic responses. This may reflect that both P2X antagonists inhibit ectonucleotidases (11), whereas suramin also blocks P2Y receptors (15). Notwithstanding this, in diabetic arteries both inhibitors displayed a greater inhibition of neurogenic responses to impulse numbers greater than two. This implies that ATP contributes more to the neurogenic response, but the extent that this is due to increased receptor function or differential transmitter release is unclear.

Phentolamine depressed responses to sympathetic stimulation in arteries from the diabetic group as we observed previ-
ously (5), and the action of exogenous norepinephrine was increased as seen by others (7, 8, 26, 28, 29, 31). Whether it made a greater contribution to sympathetically evoked responses is unclear. The contribution made by norepinephrine and by ATP separately to the evoked response (as shown by the actions of the respective antagonists) is less than their combined effects. Such synergy is greater at lower impulse numbers, but in the diabetic group this interaction is greater with higher impulse numbers. A mechanism for this synergy is unknown as is its enhancement in diabetes.

In most experiments, normal, nondiabetic injected and high glucose control group arteries responded in the same way. However, arteries from the high glucose and diabetic groups showed similar enhancements to bath-applied norepinephrine. This implies that norepinephrine and ATP actions were differentially affected by diabetes. There are several mechanisms by which acute effects of high glucose alter receptor function, from simple tonicity to shunting into signaling lipids resulting from glucose loading. Yet, high glucose produced no differences compared with noninjected in the actions of antagonists on neurogenic responses and injected groups, which infers that acute affects of high glucose were unlikely to affect endogenous neurotransmitter processes.

There were increased individual contributions to ATP and norepinephrine in the diabetic group but no overall changes in responses to neurogenic stimulation. This might be explained by additional compensatory changes. For example, ATP stimulates smooth muscle contraction but also, via P2X receptors, restricts the norepinephrine-induced contraction via its signaling pathway (3, 6, 18). Increased competition for cytosolic resources in P2X-purinergic and adrenoceptor signaling may also limit functional responses. It may be relevant in this context that P2 receptor antagonism with suramin occasionally potentiated neurally evoked responses. NF-279 inhibited neurally evoked responses to a greater degree and never caused potentiation of responses. This may reflect different potency of antagonists at P2X receptors or the possibility that suramin bound to other populations of purinergic receptors. Among...
presynaptic influences, increased release of norepinephrine is unlikely because its release is unaffected by P2 antagonists (3). Sympathetic stimulation releases a third cotransmitter NPY, and this might also interact with the postsynaptic signaling pathways. Its role in diabetes is yet to be explored.

A diabetic neuropathy and associated reduction in sympathetic vasoconstriction have been reported in the tail artery (16) and mesenteric bed (22, 25). Denervation supersensitivity is also associated with diabetic neuropathy (e.g., Refs. 8, 16, and 26). In contrast, our data with desipramine and sympathetic innervation density found no evidence for this. Thus desipramine potentiated neurogenic responses and responses to bath-applied norepinephrine in both normal and diabetic groups. If the diabetic group already had impaired norepinephrine re-uptake, this would be manifest as a smaller potentiation by desipramine, which was not the case, contrary to previous studies (8, 26). These observations again point to a diabetic modification of postsynaptic receptor/messenger coupling, although sympathetic nerve density was not changed in diabetes, a neuropathic depression in function cannot be excluded.

Although there was increased sensitivity to exogenous norepinephrine and ATP in diabetic tail arteries, sympathetically evoked responses were similar between all groups. It was possible that enhanced transmitter uptake might account for this leveling of response to neural stimulation. Our experiments found no evidence for this, although further experiments using greater impulse numbers, with concomitantly greater ATP uptake, this would be manifest as its greater role in activation of sympathetic fibers, and 3) increased potency of exogenous ATP. There was also an increased contribution by norepinephrine. These diabetic effects occurred irrespective of the stimulus pattern. These observations imply a change in receptors or cytosolic signaling pathways for these cotransmitters in diabetes.

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