

# Attenuated Purinergic Receptor Function in Patients With Type 2 Diabetes

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**OBJECTIVE**—Extracellular nucleotides and nucleosides are involved in regulation of skeletal muscle blood flow. Diabetes induces cardiovascular dysregulation, but the extent to which the vasodilatory capacity of nucleotides and nucleosides is affected in type 2 diabetes is unknown. The present study investigated 1) the vasodilatory effect of ATP, uridine-triphosphate (UTP), and adenosine (ADO) and 2) the expression and distribution of P2Y<sub>2</sub> and P2X<sub>1</sub> receptors in skeletal muscles of diabetic subjects.

**RESEARCH DESIGN AND METHODS**—In 10 diabetic patients and 10 age-matched control subjects, leg blood flow (LBF) was measured during intrafemoral artery infusion of ATP, UTP, and ADO, eliciting a blood flow equal to knee-extensor exercise at 12 W (~2.6 l/min).

**RESULTS**—The vasodilatory effect of the purinergic system was 50% lower in the diabetic group as exemplified by an LBF increase of 274 ± 37 vs. 143 ± 26 ml/μmol ATP × kg, 494 ± 80 vs. 234 ± 39 ml/μmol UTP × kg, and 14.9 ± 2.7 vs. 7.5 ± 0.6 ml/μmol ADO × kg in control and diabetic subjects, respectively, thus making the vasodilator potency as follows: UTP control subjects (100) > ATP control subjects (55) > UTP diabetic subjects (47) > ATP diabetic subjects (29) > ADO control subjects (3) > ADO diabetic subjects (1.5). The distribution and mRNA expression of receptors were similar in the two groups.

**CONCLUSIONS**—The vasodilatory effect of the purinergic system is severely reduced in type 2 diabetic patients. The potency of nucleotides varies with the following rank order: UTP > ATP > ADO. This is not due to alterations in receptor distribution and mRNA expression, but may be due to differences in receptor sensitivity. *Diabetes* 59:182–189, 2010

The net balance between vasoconstrictor and vasodilator activity in patients with type 2 diabetes is abnormal, and the ability of adjusting vascular tone during conditions with increased demands for blood flow is affected (1,2). The level of secretion and bioavailability of the potent vasodilators nitric oxide (NO) and prostacyclin (prostaglandin [PG]) are diminished, and there is an increase in the synthesis,

secretion, and action of vasoconstrictors including prostanooids, angiotensin II, endothelin, and noradrenalin (3). This leads to impaired vascular reactivity, which may limit exercise capacity in patients with type 2 diabetes.

In the last decade, the purinergic system and its impact on blood flow regulation have come more into focus. Several studies have shown that extracellular nucleotides and nucleosides such as ATP, uridine-triphosphate (UTP), and adenosine (ADO) are factors in the local control of vessel tone (4) and regulation of exercise-induced hyperemia (5). Interestingly, reduced ATP release has been shown in diabetic patients (6).

Whereas UTP and ADO are pure vasodilators, ATP possesses the ability to induce both vasodilatation and vasoconstriction; ATP-induced vasodilatation is assumed to be a result of intravascular release of ATP from endothelial cells or erythrocytes, leading to release of NO and PG via endothelial purinergic receptors (7,8), whereas extravascular ATP leads to vasoconstriction by direct action on receptors on smooth muscle cells (9). Nucleotides and nucleosides mediate a biological response via two main categories of cell surface receptors: P1 receptor for ADO and P2 receptor recognizing primarily ATP, UTP, ADP, and uridine-diphosphate. P2 receptors are subdivided into P2X and P2Y, based on their pharmacological properties; P2Y is a family of G-protein-coupled receptors, whereas P2X is a group of ligand-gated ion channel receptors (10,11).

Attenuated function of purinergic receptors is implicated in multiple diseases (12). The aim of the present study was therefore to evaluate whether the endothelial dysfunction in type 2 diabetic patients is associated with impairment of the vascular sensitivity to extracellular nucleotides and nucleosides. We hypothesized that purinergic-mediated vasodilation is diminished in type 2 diabetic patients because of decreased receptor density and/or sensitivity. We determined localization of P2Y<sub>2</sub> and P2X<sub>1</sub> receptors and measured levels of mRNA of P2Y<sub>2</sub> receptors to investigate potential downregulation.

## RESEARCH DESIGN AND METHODS

Ten subjects with type 2 diabetes and 10 healthy subjects participated in the study. All participants were fully informed of risks and discomforts associated with the experiment before giving informed written consent. The study was approved by the ethics committee in Copenhagen County and conducted in accordance with the guidelines of the Declaration of Helsinki. The diabetic patients were recruited from outpatient clinics and selected by World Health Organization criteria (Table 1).

On the day of the experiment, the subjects arrived at the laboratory after a light breakfast, refraining from alcohol and caffeine for >12 h. The diabetic subjects maintained their usual antidiabetic treatment. Before the trials, BMI and leg mass were calculated from whole-body dual-energy X-ray absorptiometry scanning (Prodigy; GE Medical Systems, Waukesha, WI).

**Experimental design.** The subjects did a pretest of one-legged knee extension (2–4 min) at 12 W (200g and 60 rotations per min) to determine the

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TABLE 1  
Demographic data for 10 healthy volunteers and 10 diabetic patients

|                                   | Control subjects | Diabetic patients |
|-----------------------------------|------------------|-------------------|
| Male/female subjects ( <i>n</i> ) | 7/3              | 7/3               |
| Age (years)                       | 51 ± 2           | 53 ± 3            |
| Body weight (kg)                  | 77 ± 3           | 96 ± 6*           |
| BMI (kg/m <sup>2</sup> )          | 24 ± 1           | 30 ± 1*           |
| Total leg mass (kg)               | 14.4 ± 0.5       | 15.8 ± 0.8        |
| Leg muscle mass (kg)              | 9.8 ± 0.5        | 10.1 ± 0.6        |
| Ghb (%)                           |                  | 7.8 ± 0.5         |
| Cholesterol (mmol/l)              | 4.7 ± 0.3        | 4.3 ± 0.6         |
| HDL cholesterol (mmol/l)          | 1.7 ± 0.2        | 1.4 ± 0.3         |
| LDL cholesterol (mmol/l)          | 2.7 ± 0.2        | 2.5 ± 0.5         |

Data are means ± SE unless otherwise indicated. \**P* < 0.01 different from control.

individual rise in leg blood flow (LBF) to this workload. The measured LBF was the target flow during the following infusions of nucleotides.

After the pretest, catheters were placed into the femoral artery and vein of the experimental leg and into the femoral artery of the nonexperimental leg under local anesthesia (5 μg/ml lidocain; Region Hovedstadens Apotek). A muscle biopsy was obtained from the middle portion of the vastus lateralis muscle using the percutaneous needle biopsy technique with suction.

Endothelial function was described by the vasodilatory response to intra-arterial infusion of acetylcholine (10 mg/ml Miochol-E; Novartis). During this test, the subjects were in a supine position to avoid an effect on cardiac output as a result of reduced preload. An LBF response <1.5 l/min to 320 μg/min (~50% reduction compared with that in control subjects) was defined as endothelial dysfunction. One diabetic participant was excluded due to a response higher than 1.5 l/min.

After 20 min of rest, the subjects were seated in the one-legged knee-extensor ergometer. In random order, infusions of ATP (A7699; Sigma), UTP (U6625; Sigma), and ADO (Item Development, Stocksund, Sweden) were performed; all drugs were dissolved in isotonic saline and infused into the femoral artery of the experimental leg. Infusion rates were increased until LBF reached a steady state at the target LBF determined during the pretest, and the infusion rate was maintained for 8 min. Ultimately, 8 min of one-legged exercise at 12 W was performed. The subjects rested at least 20 min after each intervention.

A scheduled test with nitroglycerin had to be renounced due to massive side effects. The infusion rate required to obtain an LBF similar to the exercise-induced LBF resulted in substantial decrease in mean arterial pressure (MAP) and severe headache in the first two subjects studied.

For the substances used, we titrated the amount needed to elicit an LBF similar to LBF during exercise (Fig. 1).

**Experimental techniques.** LBF was measured with ultrasound Doppler (Vivid 7; GE Healthcare) probe 8C. The two-dimensional frequency was ~6.7 MHz and the pulsed wave frequency ~5 MHz. During rest, the diameter of the femoral artery was determined under a perpendicular insonation angle as an average of three measurements (13). The site of blood velocity measurements in the common femoral artery was distal to the inguinal ligament but above the bifurcation into the superficial and profound femoral branch to avoid turbulence from the bifurcation. At this position, proximal to the exercising muscles, the vessel diameter is unaffected by contraction and relaxation (13). All recordings were obtained below an insonation angle of 60°. Sample volume was placed in the center of the vessel, adjusted to the width of the diameter, and kept clear of the vessel walls. Sample volume varied between 4.4 and 6.3 mm. Low-velocity filter (velocities <1.8 m/s) rejected noises caused by turbulence at the vascular wall.

Blood velocity was measured at baseline and after 4 and 8 min of infusion or exercise. Each time recording was obtained during three intervals of at minimum eight heart cycles. The spectrum was autotraced to standardize the calculations. LBF was calculated by multiplying the cross-sectional area of the femoral artery with the amplitude,  $V_{\text{mean}}$ , and the three measurements were averaged.

Heart rate was recorded with an electrocardiogram, and MAP was monitored with transducers positioned at heart level (Pressure Monitoring Kit; Baxter, Deerfield, IL); data were continuously recorded using a powerlab system (ADInstruments, Sydney, Australia).

Blood samples were collected at baseline and after 4 and 8 min of exercise or infusion in steady state and at the target LBF. Blood samples were obtained

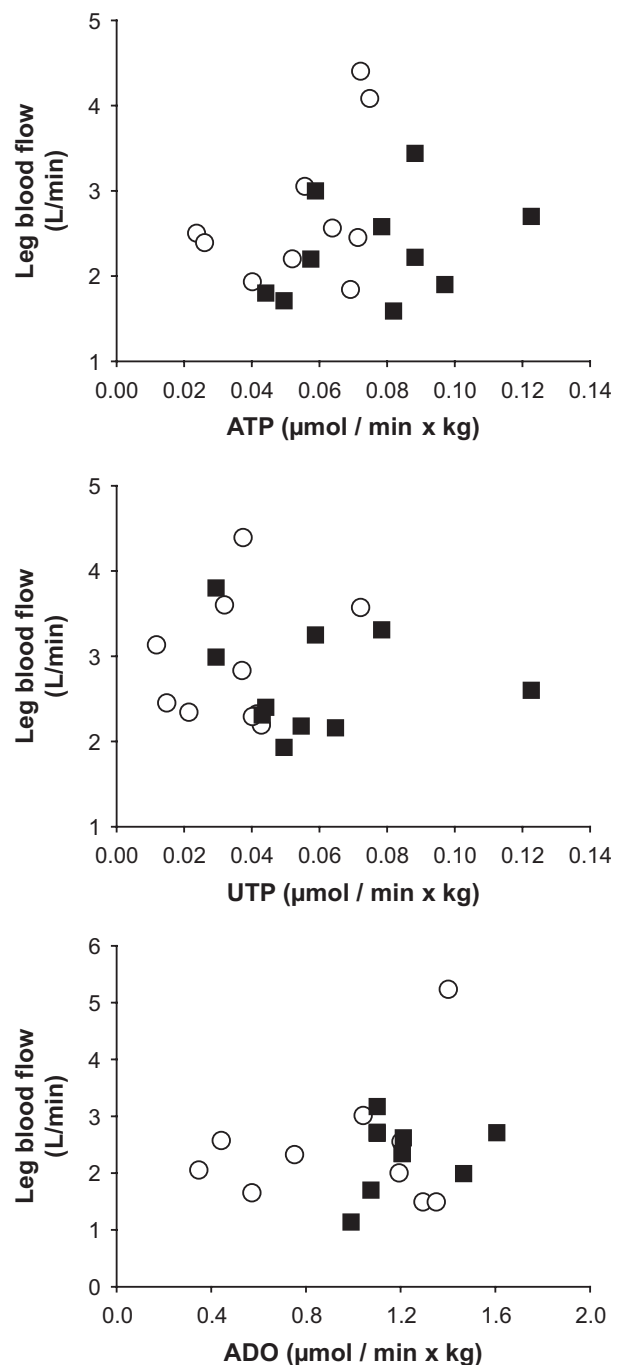
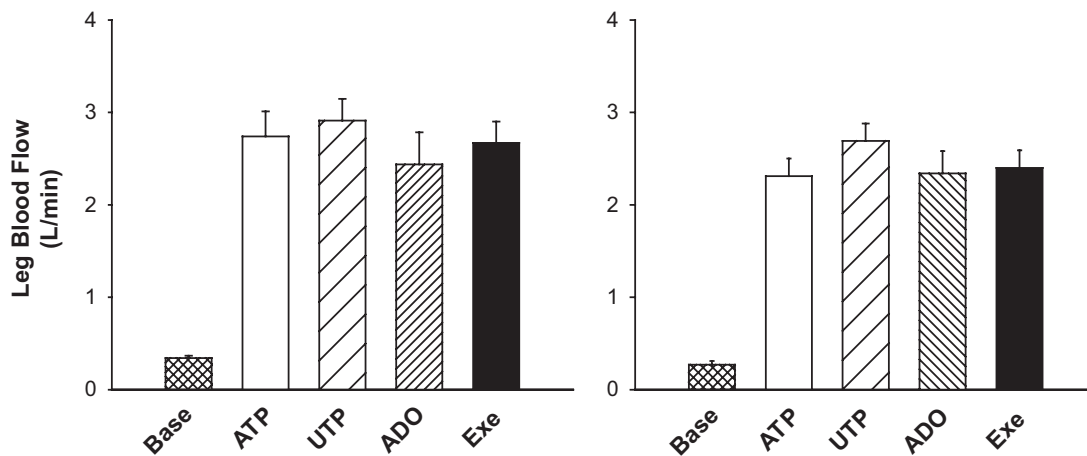


FIG. 1. Scatter plots of LBF per micromole of infused ATP, UTP, and ADO, respectively, normalized for leg total mass. Infusion rates were carefully titrated to elicit an LBF, similar to LBF during exercise. ○, Control subjects; ■, diabetic subjects.

from the femoral artery of the nonexperimental leg and with a 5-s delay from the femoral vein of the experimental leg. Blood gases and hemoglobin were measured using an ABL725 analyzer (Radiometer, Copenhagen, Denmark).

Muscle biopsies were separated in two parts: one part was quickly frozen in liquid nitrogen (<15 s) and the other part was fixed in Tissue-Tek (Sakura, Torrance, CA) and carefully frozen in isopentane, placed in a cup in liquid nitrogen. Both parts of the biopsies were stored at -80°C until they were analyzed.

**Quantification of P2Y<sub>2</sub> receptors.** Total RNA was isolated from ~20 mg of muscle tissue by a modified guanidinium thiocyanate-phenol-chloroform extraction method adapted from Chomczynski and Sacchi (1987) as previously described (14). Superscript II RNase H<sup>-</sup> system (Invitrogen, Carlsbad, CA) and oligodeoxythymidylic acids were used to reverse transcribe the mRNA to cDNA. The cDNA samples were diluted in nuclease-free H<sub>2</sub>O to a



**FIG. 2.** LBF responses showing that LBF was increased during infusions of ATP, UTP, and ADO to match LBF during exercise (Exe). The dose needed to elicit the target LBF differed substantially. In the control group (*left panel*), the mean infusion rates were as follows: ATP, 0.8  $\mu\text{mol}/\text{min}$  (range 0.4–1); UTP, 0.5  $\mu\text{mol}/\text{min}$  (range 0.2–1); and ADO, 13.7  $\mu\text{mol}/\text{min}$  (range 5–18). In the diabetic group (*right panel*), mean infusion rates were as follows: ATP, 1.2  $\mu\text{mol}/\text{min}$  (range 0.5–2); UTP, 0.9  $\mu\text{mol}/\text{min}$  (range 0.5–2); and ADO, 19.4  $\mu\text{mol}/\text{min}$  (range 17–27).

total volume of 150  $\mu\text{l}$ . The amount of single-stranded DNA was determined in each cDNA sample using OliGreen reagent (Molecular Probes, Leiden, the Netherlands) as previously described (15).

The  $\text{P2Y}_2$  mRNA content was determined by PCR using the fluorogenic 5' nuclease assay with TaqMan probes (ABI PRISM 7900 Sequence Detection System; Applied Biosystems, Foster City, CA). Forward (5' CAGCAGCGT GCTCTGCAA 3') and reverse (5' CGCTGATGCAGGTGAGGAA 3') primers and TaqMan probe (5' TGCGCTTCCTCTTCTACACCAACCTTTACTG 3') were designed and optimized for the PCR (16). The obtained critical threshold values reflecting the initial content of  $\text{P2Y}_2$  mRNA in the samples were converted to an arbitrary amount using a standard curve obtained from a serial dilution of a representative pooled sample. For each reverse transcription sample, the amount of  $\text{P2Y}_2$  cDNA was normalized to the total cDNA content.

**Immunohistochemical analysis of  $\text{P2Y}_2$  and  $\text{P2X}_1$  receptors.** The cellular localizations were determined on 8- $\mu\text{m}$  transverse sections of frozen skeletal muscle samples, incubated with a rabbit polyclonal  $\alpha\text{P2Y}_2$  antibody (APR-010; Alomone Laboratories, Jerusalem, Israel). Endothelial cells were detected with a primary monoclonal antibody, mouse  $\alpha\text{-CD31}$  (clone JC70A; DAKO, Glostrup, Denmark) and smooth muscle cells with  $\alpha\text{-smooth muscle cell actin}$  (m3558; DAKO) and then incubated with either biotin-coupled goat  $\alpha\text{-rabbit}$  (E0432; DAKO) or biotin-coupled rabbit  $\alpha\text{-mouse}$  antibody (E0354; DAKO). Staining of purinergic receptors, endothelial cells, and smooth muscle cells was performed on serial sections. Antibody binding was visualized with streptavidin coupled to fluorescein isothiocyanate (FITC). Negative control subjects were achieved without the primary antibody. Immunoreactive cells were examined and photographed in a Zeiss Axioplan Microscope (Carl Zeiss, Jena, Germany) (7).

**Calculations.** Cardiac output was calculated from arterial blood pressure using the model flow method (Beat Scope 1.1a; Finapres Medical Systems BV, Amsterdam, the Netherlands). Stroke volume was calculated as the quotient between cardiac output and heart rate. Leg vascular conductance (LVC) was calculated as the quotient between LBF and MAP. Leg oxygen delivery was calculated as the product of arterial oxygen content and LBF. Leg oxygen uptake ( $\text{VO}_2$ ) was calculated as the product of the arteriovenous (a-v) difference of oxygen and LBF.

**Statistical analysis.** Baseline values were tested using a Student *t* test.  $\delta$ -Values of the hemodynamic variables were analyzed by a two-way repeated-measures ANOVA, with nucleotides and control/type 2 diabetes as between-subject factors. If normality or equal variance failed, data were log-transformed. There was no difference in any variable between 4 and 8 min of infusion or exercise; therefore, only data after 8 min are presented. The significance level was set at  $P < 0.05$ . Data are presented as means  $\pm$  SE unless otherwise stated.

## RESULTS

**Flow responses and vasodilator potency.** Both leg muscle mass and leg total mass were comparable in control versus diabetic subjects (Table 1). In addition, the resting LBF was similar between the two groups ( $0.34 \pm$

$0.02$  vs.  $0.27 \pm 0.03$  l/min) (Fig. 2) as was the flow response to 12 W of workload ( $2.7 \pm 0.2$  vs.  $2.4 \pm 0.2$  l/min). However, the increase in LBF per micromole of infused substance was different for control versus diabetic subjects:  $274 \pm 37$  vs.  $143 \pm 26$  ml/ $\mu\text{mol}$  ATP  $\times$  kg,  $494 \pm 80$  vs.  $234 \pm 39$  ml/ $\mu\text{mol}$  UTP  $\times$  kg, and  $15 \pm 3$  vs.  $7.5 \pm 0.6$  ml/ $\mu\text{mol}$  ADO  $\times$  kg (Fig. 1). Thus, the vasodilator potency was as follows: UTP control subjects (100) > ATP control subjects (55) > UTP diabetic subjects (47) > ATP diabetic subjects (29) > ADO control subjects (3) > ADO diabetic subjects (1.5). Within the groups, the potency varied with similar order of rank and proportions: UTP (100) > ATP (55) > ADO (3) for control subjects versus UTP (100) > ATP (61) > ADO (3.2) for the diabetic subjects.

**Mean arterial pressure and leg vascular conductance.** MAP was similar in the two groups at baseline ( $109 \pm 6$  vs.  $99 \pm 4$  mmHg for control subjects vs. diabetic subjects, respectively) (Fig. 3). In both groups, MAP decreased during all three infusions by 5–10 mmHg, with the largest decrease during ATP infusion. During exercise, MAP increased similarly ( $125 \pm 12$  vs.  $124 \pm 5$  mmHg).

LVC was similar at baseline ( $3.0 \pm 0.2$  vs.  $2.7 \pm 0.3$  ml  $\cdot$  min $^{-1}$   $\cdot$  mmHg $^{-1}$  for control subjects vs. diabetic subjects, respectively), during exercise ( $23 \pm 2$  vs.  $21 \pm 2$ ), and during the three infusions, increasing in the range of 26 to 30.

**Oxygen delivery and uptake.** Oxygen delivery to the leg did not differ between the groups at baseline ( $59 \pm 6$  vs.  $49 \pm 4$  ml/min) or during infusion or exercise (range 444–552 ml/min).

The a-v  $\text{O}_2$  difference was larger in the diabetic subjects at baseline ( $78 \pm 5$  vs.  $100 \pm 6$  ml/l). During the three infusions, there were no differences in the groups, but during exercise, a-v  $\text{O}_2$  difference was larger in the diabetic group ( $129 \pm 4$  vs.  $142 \pm 6$  ml/l).

The larger a-v difference in the diabetic group compensated for a slightly lower LBF; both groups had a  $\text{VO}_2$  of  $\sim 25$  ml/min at baseline and  $\sim 360$  ml/min during exercise. During the three infusions,  $\text{VO}_2$  ranged between 28 and 45 ml/min with no difference between the groups.

Venous lactate differed at baseline ( $1.0 \pm 0.2$  vs.  $2.0 \pm 0.3$  mmol/l), but there was no difference in the increase during exercise ( $\sim 1.6$  mmol/l) or in the decrease during the infusions (0.1–0.3 mmol/l).

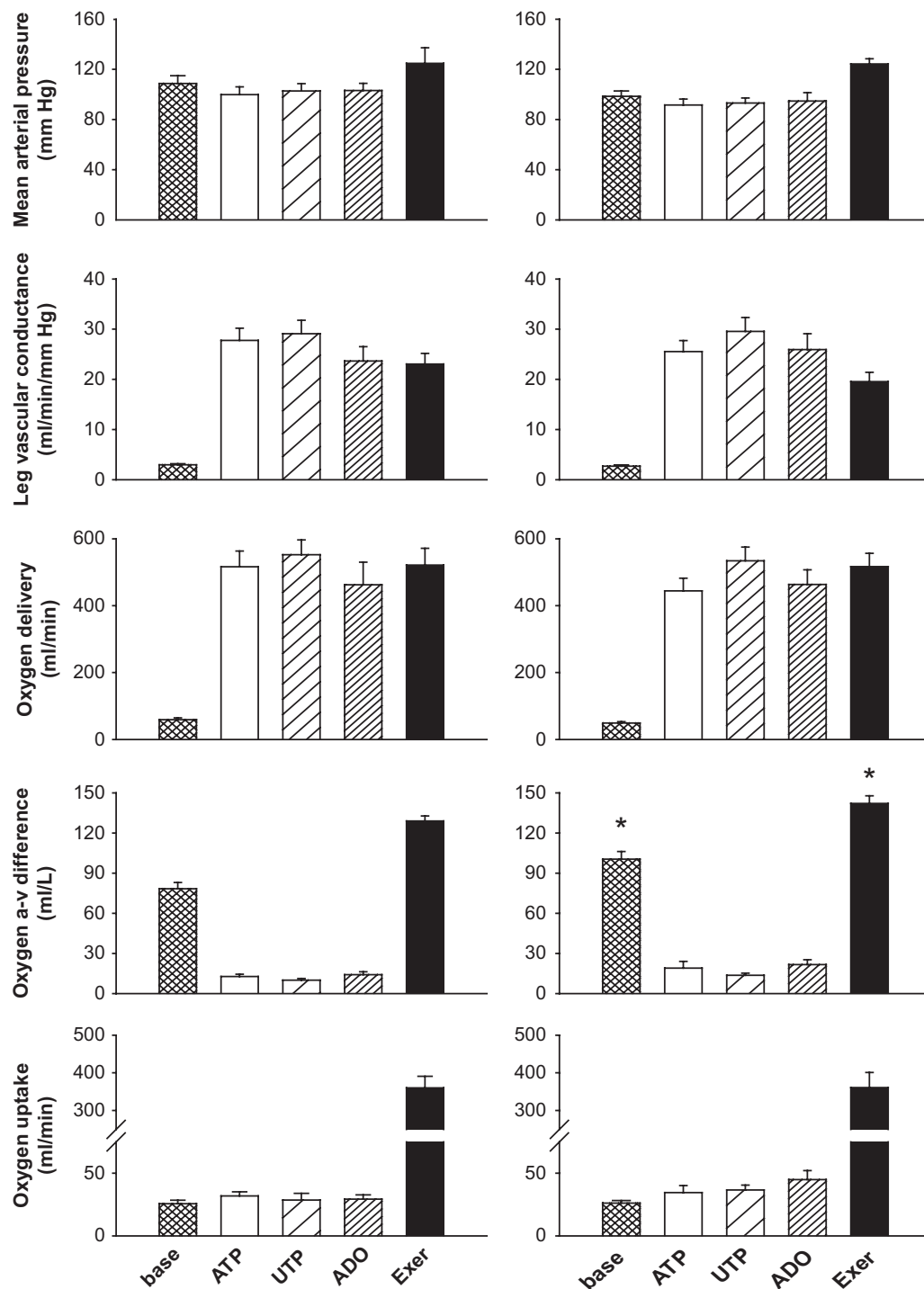


FIG. 3. Local hemodynamic variables at baseline; during infusions of ATP, UTP, and ADO; and during exercise (Exer) in control (left panel) and diabetic (right panel) subjects. \* $P < 0.05$  compared with control subjects.

**Central cardiovascular response.** Heart rate at baseline tended to be lower for the control group than the diabetic group ( $67 \pm 3$  vs.  $77 \pm 4$  bpm,  $P = 0.056$ ) (Fig. 4). Heart rate increased by  $\sim 5$  bpm during infusions, independent of group and nucleotide. During exercise, heart rate increased to similar levels for the two groups ( $92 \pm 5$  vs.  $95 \pm 5$  bpm for control subjects vs. diabetic subjects, respectively).

Stroke volume was different at baseline ( $70 \pm 3$  vs.  $90 \pm 5$  ml/beat for control subjects vs. diabetic subjects, respectively). The elevation of stroke volume during the infu-

sions was similar in the two groups and increased in the range of 10–17 ml/min and was essentially unchanged during exercise.

Consequently, cardiac output also differed at baseline ( $4.7 \pm 0.2$  vs.  $6.8 \pm 0.5$  l/min for control subjects vs. diabetic subjects, respectively). There was, however, no difference in the increase of cardiac output between the two groups during the various interventions, although the small increase in cardiac output during exercise showed a tendency to be higher in the diabetic group ( $P = 0.056$ ).

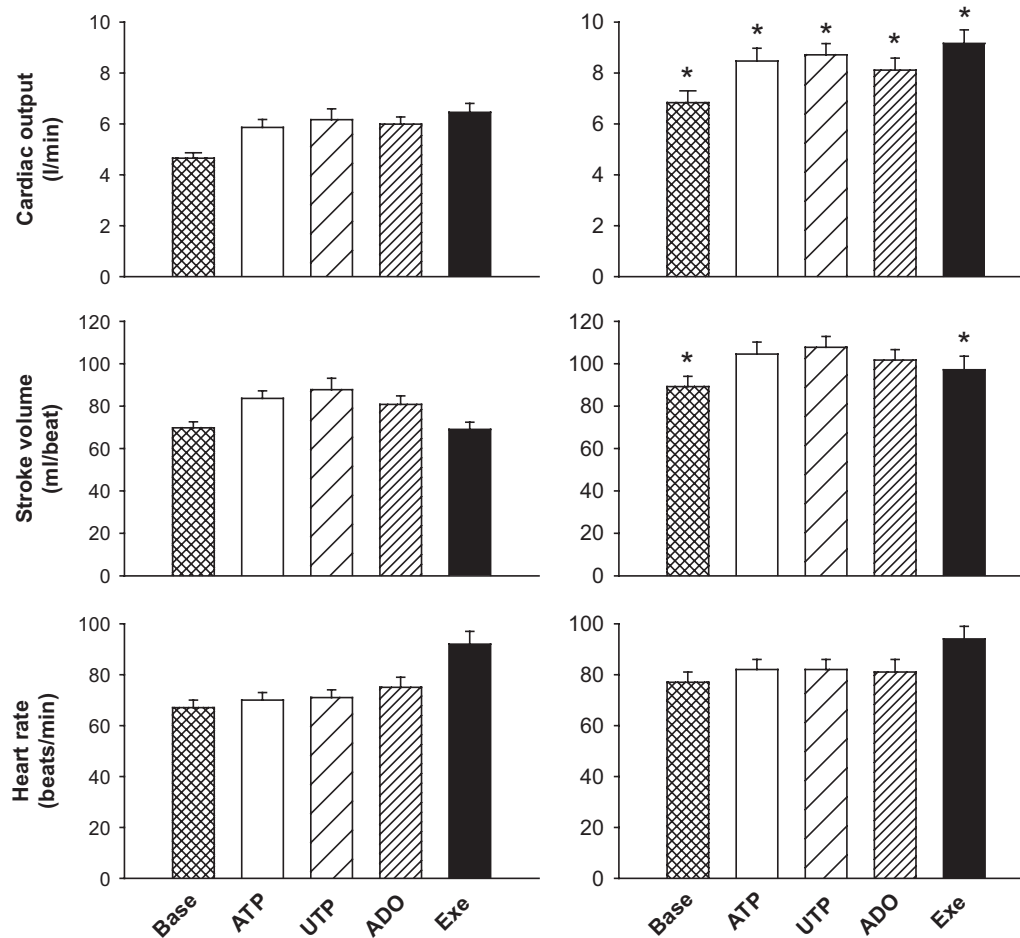


FIG. 4. Systemic hemodynamic variables at baseline; during infusions of ATP, UTP, ADO; and during exercise (Exe) in control (left panel) and diabetic (right panel) subjects. \* $P < 0.05$  compared with control subjects.

**Receptor distribution and quantity.** The distribution of P2Y<sub>2</sub> and P2X<sub>1</sub> receptors was identical in the two groups. P2Y<sub>2</sub> receptors were located in the endothelium of capillaries and in endothelium and smooth muscle cells of microvessels. The P2X<sub>1</sub> receptors were present in the endothelium of capillaries and in the skeletal muscle sarcolemma but not in smooth muscle cells (Fig. 5).

Also, the quantity of mRNA for P2Y<sub>2</sub> receptors was similar in the two groups ( $69 \pm 15$  vs.  $70 \pm 16$  arbitrary units for control subjects vs. type 2 diabetic subjects, respectively).

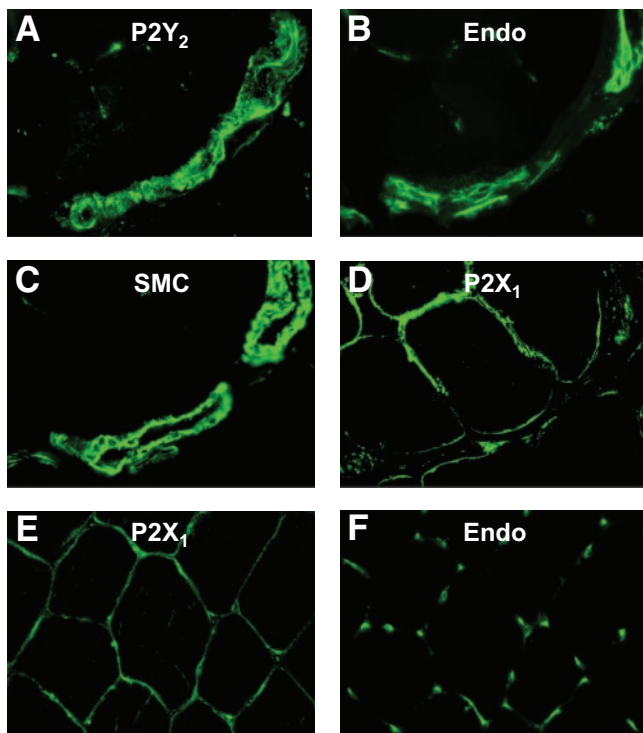
## DISCUSSION

The main findings of the present study are that 1) the vasodilatory response to ATP, UTP, and ADO infusion was lower in patients with type 2 diabetes compared with age-matched control subjects and 2) type 2 diabetes does not affect the distribution or the gene expression of P2Y<sub>2</sub> or P2X<sub>1</sub> receptors.

Nucleotides have been found to play a role in the regulation of skeletal muscle blood flow during hyperemic conditions, including exercise, where shear stress and hypoxia are important stimuli of both ATP and UTP release from endothelial cells. Erythrocytes are proposed to function as oxygen sensors, contributing to the muscle blood flow regulation by the simultaneous release of ATP and S-nitrosohemoglobin in proportion to off-loading O<sub>2</sub> from the hemoglobin molecule, thereby inducing vasodi-

lation and a consequential rise in oxygen delivery to the tissues in demand (17,18). However, erythrocytes from humans with type 2 diabetes, incubated with the stimulating agent Mastoparan 7, released less ATP than erythrocytes from nondiabetic subjects (6). The vasodilatory effect of ATP, UTP, and ADO has so far been tested only in young healthy subjects with a normal endothelial function. In the current study, all diabetic subjects had endothelial dysfunction as evidenced by a reduction in LBF during acetylcholine infusion, and needed a higher dose of nucleotides, in general twice as much, to elicit a rise in leg blood flow similar to that achieved during moderate exercise. These findings indicate that type 2 diabetes affects the purinergic system, compromising cardiovascular regulation in diabetic patients. It is, therefore, likely that the reduced exercise capacity of diabetic patients is due partly to less available ATP in combination with a reduction in vasodilatory potency of ATP.

The mechanisms underlying these findings may be several. In young healthy subjects, pharmacological inhibition of both NO and PG synthesis during ATP infusion has been found to diminish leg blood flow by 40%, indicating that the vasodilatory effect of ATP is mediated by NO and PG but also by additional mechanisms (7). Similarly, inhibition of both NO and PG synthesis during ADO infusion has been reported to reduce blood flow by ~70%, with a larger reduction occurring during PG blockade compared with NO blockade, suggesting that to a large extent, the vaso-



**FIG. 5.** Immunohistochemical localization of purinergic P2Y<sub>2</sub> and P2X<sub>1</sub> receptors in human skeletal muscle. *A–D*: Different staining on serial sections. *A*: Positive staining for P2Y<sub>2</sub> (APR-010; Alomone Laboratories). *B*: Positive staining for endothelium (Endo) (ulex europaeus agglutinin-1 [UEA-1]). *C*: Positive staining for vascular smooth muscle cells (SMC) ( $\alpha$ -smooth muscle cell actin [m3558; DAKO]). *D*: Positive staining for the P2X<sub>1</sub> receptor (APR-001; Alomone Laboratories). P2Y<sub>2</sub> purinergic receptors were present in endothelial cells of capillaries and microvessels and in vascular smooth muscle cells, whereas P2X<sub>1</sub> receptors were not present in smooth muscle cells. *E* and *F*: Positive staining for the P2X<sub>1</sub> receptor (APR-001; Alomone Laboratories) (*E*) and positive staining for endothelium (ulex europaeus agglutinin-1) (*F*) on the same section as in *E*. The P2X<sub>1</sub> receptors were present in the endothelium of capillaries and in the skeletal muscle sarcolemma. Antibody binding was visualized with streptavidin coupled with FITC.

dilatatory effect of ADO is mediated through NO and PG and that the increased NO formation could be prostaglandin mediated (19). Several studies show that type 2 diabetes diminishes the bioavailability of NO and PG and decreases the half-life and activity of NO (20,21). Consequently, part of the reduced vasodilatatory effect of the infused nucleotides and nucleoside may be attributed to a reduction in availability and activity of NO and/or prostaglandins or other mediators.

Alternatively, the impaired sensitivity to nucleotides could be related to a more extensive formation and action of vasoconstricting compounds. Diabetes is known to be associated with increased synthesis of constrictory compounds (i.e., prostanoids, endothelin, and angiotensin II); therefore, a higher constrictory tone might underlie the reduction in vasodilatatory effect of the purinergic agonists (3,22). Also, type 2 diabetic patients have a higher muscle sympathetic nerve activity and a higher sensitivity to catecholamines (21,23). Enhanced adrenergic receptor sensitivity might relate to endothelial damage because the intact endothelium attenuates adrenergic reactivity in control arteries, a mechanism that was not present in arteries from diabetic patients (24).

In contrast to NO, nucleotides are preferentially arterial dilators. They are capable of regulating local vascular tone in two complimentary ways. It was recently demonstrated

that ATP and UTP induce potent vasodilation at rest but also oppose a concomitant increase in sympathetic activity as seen during exercise, indicating that circulating nucleotides may be modulators of sympathetic vasoconstriction during exercise—a phenomenon termed functional sympatholysis (25–27). The phenomenon does not occur during ADO or NO infusion or during NO blockade during exercise, suggesting that other secondary metabolites must be of importance (28).

Despite the reduced vasodilatatory capacity of ATP, UTP, and ADO in the diabetic subjects, the response to exercise was diminished by ~10% in the diabetic group, indicating that none of the infused substances is mandatory to elicit exercise hyperemia. Most exercise studies show that blood flow will be reduced but not hindered despite multiple pathway blockades, suggesting that redundancy will occur to sustain exercise performance. In this context, ATP is not an obligatory vasodilator in normal or pathological conditions and other systems may be upregulated, exemplified in settings of reduced NO availability, for instance, in the endothelial nitric oxide synthase knockout mice, where endothelium-derived hyperpolarizing factor-mediated relaxation in resistance arteries is upregulated (29). Also, greater reliance on K<sub>ATP</sub> channels may occur (30).

In studies of young healthy subjects, ATP and UTP showed the same potency in regulation of skeletal muscle blood flow. In contrast, ATP and UTP did not have equal potency in the present study, either in the control or the diabetic group. There are some possible explanations for this. First, in vitro inhibition of NO-mediated dilatation of ATP and UTP showed that UTP exceeded ATP, indicating that UTP could be less dependent on NO (31). Moreover, a recent study on the affect of aging on skeletal muscle blood flow found that age-dependent reduction in forearm blood flow is due to a decline in endothelial-derived NO (32). Therefore, it is likely that a decrease in bioavailability and activity of NO and PG affects the potency of ATP more than the potency of UTP, leading to the differences observed in the present study. Second, the intact endothelium does not allow intraluminal ATP to pass the barrier (33), but when endothelial damage occurs, it cannot be excluded that intraluminal metabolites, including ATP, diffuse to the interstitium, activating extraluminal P2X receptors on vascular smooth muscle cells, thus resulting in a concurrent vasoconstriction. UTP does not have this constrictory ability, and endothelial leakage may, in this context, affect the vasodilatatory effect of UTP less than ATP. Also, regarding ADO, we found a change in potency in both groups compared with that in other studies of young healthy subjects (ATP:ADO = 1:16 in young subjects vs. 1:19 in both groups of the present study) (25). Again, because the effect of ADO is mediated mainly by formation of PG and NO, the impaired NO formation accompanying aging may reduce the vasodilatatory effect of ADO more than ATP and UTP, and the further reduction of effect in the diabetic group reflects the supplementary impact of diabetes on the mediators of the vasodilatatory responses.

The present study focused on the peripheral aspects of blood flow and infusions were therefore performed in the femoral artery with little effect on systemic hemodynamic parameters. The diabetic subjects had higher stroke volume and cardiac output, but both variables are calculated, and indirect measurements of cardiac output in obese subjects may be overestimated. Importantly, there were no

differences in the  $\delta$ -values. The trend of a slightly lower LBF in the diabetic subjects during exercise may be the result of a reduced exercise capacity because some diabetic subjects had difficulties fulfilling 8 min of exercise at this modest workload despite the similar supply of oxygen.

Quantification of the P2Y<sub>2</sub> receptor did not show differences in the level of gene expression in the two groups, indicating that type 2 diabetes does not affect the rate and way of mRNA expression of the P2Y<sub>2</sub> receptor gene. This does not preclude, however, that P2Y<sub>2</sub> and P2X<sub>1</sub> receptor function could be altered in diabetic patients. It is well known that pathology may induce differences in a transcribed protein, thus modifying the properties of a protein or a receptor after transcription. Immunohistochemical analysis revealed that localization of P2Y<sub>2</sub> and P2X<sub>1</sub> receptors was consistent with previous findings in young healthy subjects, allowing us to conclude that the reduction in vasodilatory potency is not due to changes in receptor distribution. Still, the analysis is a qualitative investigation and does not exclude differences in receptor amount and a potential change in function of the receptors; a profound morphological analysis of receptor amount and quality could elucidate potential differences in function and distribution of the receptors and target proteins. P2X<sub>1</sub> especially could be of interest because this receptor is widely distributed in the plasma membrane of skeletal muscle cells.

In summary, the present findings demonstrate that the vasodilatory potency of the purinergic system is attenuated in diabetic patients and of such a magnitude that it could be an important factor for the observed impairment in regulation of vascular tone in diabetic patients. Potential explanations for the reduced effect of the purinergic system could be 1) less available ATP as a result of a decline in release from erythrocytes, 2) reduced bioavailability of different mediators, 3) an intensified vasoconstrictory counteraction, and 4) an increase in sympathetic tone, resulting in impaired functional sympatholysis. The present observations suggest that the purinergic system may be involved in the pathogenesis of endothelial dysfunction and, thereby, underlie the cardiovascular complications associated with diabetes. The attenuation in purinergic receptor responses could, in part, be implicated in capillary rarefaction and impaired regulation of capillary recruitment in skeletal muscle tissue, known to compromise insulin sensitivity and glucose uptake. The current findings, in combination with existing evidence, provide a basis for further studies addressing the mechanisms underlying the reduced vascular function and exercise capacity in diabetic patients.

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