Enhanced P2X<sub>7</sub> Activity in Human Fibroblasts From Diabetic Patients

A Possible Pathogenetic Mechanism for Vascular Damage in Diabetes

Anna Solini, Paola Chiozzi, Anna Morelli, Elena Adinolfi, Roberta Rizzo, Olavio R. Baricordi, Francesco Di Virgilio

**Objective**—We have investigated expression and function of the P2X<sub>7</sub> receptor in fibroblasts from healthy subjects and patients with type 2 diabetes.

**Methods and Results**—Fibroblasts were isolated from skin biopsies. P2X<sub>7</sub> receptor expression in both cell populations was measured by functional assays, RT-PCR, fluorescence-activated cell sorter, and immunoblotting. We found that fibroblasts from diabetic subjects are characterized by enhanced P2X<sub>7</sub>-mediated responses as indicated by increased shape changes, microvesiculation, enhanced fibronectin and interleukin 6 secretion, and accelerated apoptosis. These responses were blocked by preincubation with the P2X blockers KN-62, oxidized ATP, or pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid). Furthermore, we also found a higher level of spontaneous fibronectin secretion and of apoptosis in fibroblasts from diabetic compared with healthy subjects. Both higher basal level of fibronectin secretion and spontaneous rate of apoptosis were likely attributable to the increased pericellular concentration of ATP because fibroblasts from diabetic subjects released 3× as much ATP into the supernatants compared with fibroblasts from healthy subjects.

**Conclusions**—We conclude that fibroblasts from type 2 diabetes patients are characterized by a hyperactive purinergic loop based either on a higher level of ATP release or on increased P2X<sub>7</sub> reactivity. (Arterioscler Thromb Vasc Biol. 2004;24:1240-1245.)

**Key Words:** P2 receptors ■ fibroblasts ■ atherosclerosis ■ cytokines ■ diabetes ■ apoptosis

Fibroblasts are a key structural element of the arterial wall and a target and source of several diffusible factors that regulate the homeostasis of circulating and vessel wall cells. They are well known for being the major producers of extracellular matrix, an active source of inflammatory mediators, as well as key players in wound repair and tissue remodeling. In human pathology, fibroblast dysfunction is implicated in diseases of unknown etiology, such as scleroderma, but also in chronic degenerative diseases, such as atherosclerosis or diabetic angiopathy. In the vessel wall, fibroblasts and smooth muscle cells share several features, and it is well established that within the atherosclerotic plaque, smooth muscle cells may acquire a dedifferentiated phenotype that resembles that of fibroblasts. In turn, activated fibroblasts proliferate and migrate into the plaque, contributing to plaque thickening and fibrous cap formation. In the atherosclerotic lesion, fibroblasts are the main source of extracellular matrix and the main causative agent of the progressive fibrosis, as well as an active source of mediators that stimulate endothelial cells and promote recruitment of leukocytes, thus accelerating damage of the arterial intima and media. Under certain poorly known conditions, the fibrous cap can undergo thinning and set the conditions for life-threatening plaque rupture. Molecular mechanisms underlying this dramatic outcome are basically unknown, but sudden apoptosis of the fibrous cap cell layer might have an important triggering role.

Receptors for extracellular nucleotides (P2 receptors) are a focus of increasing attention in vascular biology and pathology. Extracellular ATP is known to cause vasodilation, probably mediated by NO release, or alternatively, contraction, probably mediated by direct smooth muscle cell stimulation. In addition, ATP induces cytokine secretion, chemotaxis of inflammatory cells, smooth muscle cell proliferation, or cytotoxicity. Among other nucleotides, ADP has a very important role in vascular biology for its powerful platelet-aggregating action.

Effects of extracellular nucleotides are mediated via activation of 2 families of distinct cell surface receptors, P2X and P2Y. We recently investigated expression and function of...
the P2X<sub>7</sub> receptor in human fibroblasts and have discovered that P2X<sub>7</sub>-mediated responses in human fibroblasts are potentiated when these cells are cultured in the presence of high glucose, a condition that is an in vitro mimic of hyperglycemia. The P2X<sub>7</sub> receptor is the most intriguing P2 receptor for its ability to undergo a channel-to-pore transition that generates a nonselective plasma membrane pore permeant to hydrophilic molecules of molecular mass up to 900 kDa.21,22 Furthermore, this receptor is well known for its potent cytotoxic activity<sup>23,24</sup> and its ability to mediate massive release of interleukin 1β (IL-1β).<sup>25,26</sup> In the present work, we extended the investigation of P2X<sub>7</sub>-mediated responses to fibroblasts from type 2 diabetes (T2D) patients. Our data show that P2X<sub>7</sub>-dependent responses are enhanced in T2D compared with control fibroblasts, even when grown at physiological glucose concentration.

**Methods**

The Methods section is available online at http://atvb.ahajournals.org.

**Results**

**Differential Sensitivity of Fibroblasts From Healthy and Diabetic Subjects**

Functional expression of the P2X<sub>7</sub> purinoceptor can be detected by low molecular weight dye uptake assays based on the typical feature of this receptor, which forms a poorly selective pore when activated.<sup>21,27,28</sup> Figure I (available online at http://atvb.ahajournals.org) shows fluorescence microcopy of fibroblasts of T2D and healthy subjects treated with 2 mmol/L ATP in the presence of the fluorescent dye YO-PRO. T2D fibroblasts showed an increased YO-PRO uptake compared with healthy cells (compare Figure IE and IG). Fluorescence intensity of individual cells from 3 different microscopic fields was quantitated in arbitrary units by image analysis with MetaMorph (Universal Imaging Corp.). Values in arbitrary fluorescence units (FU) were (average±SD) 68.42±28.30 (n = 35) and 159.80±39.15 (n = 65) for fibroblasts from healthy and T2D subjects, respectively (P < 0.001; Student t test or ANOVA). Similar results were obtained at a lower concentration (0.5 mmol/L) of the more potent P2X<sub>7</sub> agonist benzoylbenzoyl ATP (BzATP) in fibroblasts from healthy and T2D subjects incubated in the same conditions reported in Figure I (data not shown). We showed previously that although P2X<sub>7</sub> activation usually causes swelling, blebbing, microvesicle formation, and death of most cell types, in human fibroblasts, these morphological changes are remarkably delayed or even absent.<sup>20,29</sup> In marked contrast, T2D fibroblasts were sensitive to ATP. This nucleotide at a concentration of 1 mmol/L mainly caused changes in cell shape (swelling followed by shrinkage), with little cytoplasmic microvesicle formation (Figure 1, compare 1C with 1D). Conversely, in the presence of a low BzATP concentration (0.5 mmol/L), swelling of T2D fibroblasts was massive and paralleled by a dramatic cytoplasmic microvesiculation (compare Figure IE and IF). At higher concentrations (1 mmol/L), BzATP caused rapid and irreversible shrinkage (compare Figure 1G and 1H). Quantitative analysis of several monolayers revealed that the near totality of T2D fibroblasts exposed to BzATP contained microvesicles and that the average number of microvesicles per cell±SD was 75±25 (n = 85). T2D cells that did not contain microvesicles were frankly apoptotic. Very few cells from healthy controls (<5%) underwent microvesiculation, and microvesicle content was 10±5 per cell (n = 55). These changes were inhibited fully by pretreatment for 2 hours with 0.3 mmol/L oxidized ATP (oATP; data not shown). The ATP and BzATP doses effective on T2D fibroblasts were reported previously to cause morphological alterations in other cell types.<sup>17,21,30</sup> At variance with ATP and BzATP, ADP, UTP, UDP, and CTP were ineffective. Furthermore, 5-hour incubation of T2D but not control fibroblasts in the presence of 5 mmol/L ATP caused full-blown apoptosis (data not shown).

**Release of Secretory Products**

Fibronectin is a secretory product that accumulates in the extracellular matrix caused by several disease conditions, among which diabetes is most notable.<sup>31</sup> Under resting conditions, fibroblasts show little intracellular staining for fibronectin (Figure 2C and 2G); however, 1 hour of ATP stimulation caused a large increase in fluorescence localized mainly in the perinuclear region (Figure 2E and 2I) and likely corresponding to the endoplasmic reticulum and Golgi apparatus (also see reference 29). Fluorescence intensity in arbitrary units, quantitated as in Figure 1, was 102.50±35 (n = 35) and 27.30 for healthy (n = 27.30) and T2D (n = 27.30) fibroblasts, respectively (P < 0.01). Enhanced intracellular accu-
mulation of fibronectin was paralleled by an increased extracellular release (Figure 3A). Quiescent fibroblasts released a small amount of fibronectin whether they originated from T2D or healthy subjects, but 1 hour of stimulation with ATP or BzATP triggered much more fibronectin secretion in T2D. We reported in a previous study that ATP was a stimulus for release of the cytokine IL-6 in fibroblasts from healthy subjects primed with lipopolysaccharide (LPS) and phorbol myristate acetate (PMA). Here we show that T2D fibroblasts release about twice as much IL-6 compared with fibroblasts from healthy subjects at any ATP concentration tested (Figure 3B). Among other nucleotides tested, BzATP at a concentration of 0.05 mmol/L caused an IL-6 release similar to that induced by 0.1 mmol/L ATP, whereas the IL-6 release caused by 0.1 mmol/L UTP was ~30% of that triggered by ATP. ATP-stimulated cytokine release was substantially but not completely (70% to 80%) blocked by preincubation in the presence of 300 μmol/L oATP or 50 nM KN-62 (data not shown). Incomplete blockade by these inhibitors and partial stimulation by UTP suggest that other P2 receptors besides P2X7 may also mediate ATP-dependent IL-6 secretion. Interestingly, even in the absence of added ATP, T2D but not control fibroblasts secreted IL-6. We hypothesized that the higher spontaneous fibronectin and IL-6 secretion was caused by local ATP release, which, in turn, fueled an ATP-based autocrine–paracrine loop, keeping most cells under constant basal stimulation. In support of this hypothesis, in the absence of any overt perturbation, T2D fibroblasts accumulated an ATP amount at least 3× higher than controls (0.25±0.06 and 0.07±0.02 μg of ATP/10^6 cells for T2D and control fibroblasts, respectively) in the extracellular space. To test whether spontaneous ATP release could support basal fibronectin secretion, we measured fibronectin accumulation during 5 hours of culture in the presence of apyrase, a soluble ATPase/ADPase. As shown in Figure 4,
shows that both ATP and BzATP caused a much larger collapse of plasma membrane potential in T2D than control fibroblasts and a leftward shift in dose dependency, especially with BzATP. The ATP and BzATP dose-dependency curves in T2D fibroblasts were shifted further to the left by preincubation with the ATP/ADP-hydrolizing enzyme apyrase (Figure IVc and IVd).

Discussion
Fibroblasts are a key component of the vessel wall known to play a major role in diabetic angiopathy and atherosclerosis.5,6,32,33 They proliferate in arterial wall and are the main source of extracellular matrix that causes the progressive fibrosis of the plaque. Furthermore, they participate in activation of endothelial cells and recruitment of leukocytes.3,4 In diabetes, the arterial wall undergoes accelerated degenerative changes (diabetic angiopathy),34 the pathogenesis of which is incompletely understood but that undoubtedly implicates profound modifications of fibroblast reactivity. Secretion of a host of inflammatory factors is known to be increased in diabetes,35 and several of these factors modify fibroblast responses. Reports in the literature suggest that in diabetic patients, fibroblast responses might be inherently aberrant,36,37 thus making these cells a very sensitive target of inflammatory factors released into the blood or the arterial wall.

In recent years, several laboratories, including our own, have suggested a role in inflammation for a novel mediator: extracellular ATP.21,38,39 Nowadays, it is a well-established fact that this nucleotide plays an important function as an extracellular signaling molecule in the central and peripheral nervous systems, in platelet aggregation, or in vasodilation.40,41 However, it is less appreciated that ATP profoundly affects immune and inflammatory cell functions as well as fibroblast responses. ATP promotes key proinflammatory responses such as leukocyte chemotaxis,15 NO generation,12 nicotinamide-adenine dinucleotide phosphate oxidase activation,42 cytokine release,14 or cytotoxicity.17 Furthermore, it is likely that ATP is released at the site of atherosclerotic lesions or during platelet adhesion to the endothelium.43

We have shown previously that primary fibroblasts from healthy subjects react to stimulation with ATP with striking morphological alterations and an increased formation of cytoplasmic microvesicles.29 In addition, they also release IL-6, provided that they are primed with LPS and PMA. These responses are potentiated dramatically in vitro by incubation in the presence of high (22 mmol/L) glucose concentration.20 Under these conditions, P2X7 receptor expression is not grossly changed, but its activity is enhanced. This observation may suggest that environmental conditions in diabetes play an important role in the mechanism of tissue damage typical of this disease. In support of this hypothesis, in this study, we show that even in the presence of a physiological glucose concentration (5.5 mmol/L), fibroblasts from T2D patients show enhanced P2X7-mediated responses. Fibroblasts from healthy individuals do not permeabilize well in response to ATP because, as we have documented previously, in these cells, uptake of normally impermeant hydrophilic solutes (eg, lucifer yellow or YO-PRO) that is gener-
ally considered the hallmark of P2X<sub>7</sub> function is usually delayed and of low intensity. On the contrary, in T2D fibroblasts, YO-PRO uptake is fast and extensive. Likewise, the peculiar microvesicles we described previously in ATP-stimulated normal fibroblasts form earlier and are of larger size in T2D fibroblasts. In a previous article, we assigned these vesicles to the Golgi compartment but made no attempt to identify any particular secretion product relevant for diabetic angiopathy. We report in this study that these large cytoplasmic vesicles contain fibronectin, known to be a main constituent of the extracellular matrix that accumulates in the interstitial space (arterial wall, mesangium, etc) in diabetes, and it is believed to play a major role in the pathogenesis of diabetic tissue damage. These vesicles are part of a secretory pathway because a large amount of fibronectin is also secreted into the extracellular space. The other secretory product relevant for diabetic angiopathy is IL-6. T2D fibroblasts release about twice as much IL-6 compared with cells from healthy controls even under resting conditions (ie, in the absence of added ATP). Secretion is increased further by ATP. Dose dependency and pharmacology of the response strongly implicate P2X<sub>7</sub> as the receptor involved. Not surprisingly, T2D fibroblasts, in striking contrast to those of control subjects, are also strongly susceptible to ATP-mediated apoptosis.

In the absence of added nucleotides, fibroblasts from diabetic patients released a higher amount of fibronectin and underwent a higher level of apoptosis. Furthermore, these cells also showed a higher basal ATP release. Ability of apyrase to reduce both basal fibronectin release and spontaneous apoptosis indicates that these responses are at least in part dependent on autocrine stimulation of P2X<sub>7</sub> by secreted ATP. This receptor has a low affinity for ATP, thus one wonders whether the nucleotide concentrations measured in the supernatants are sufficient for activation. However, it is clear that the ATP levels measured by us are only grossly indicative of the real ATP concentration at the level of the plasma membrane. If basal ATP release is indeed sufficient to cause P2X<sub>7</sub> activation, then we might hypothesize that the higher sensitivity of T2D cells to ATP could be attributable to an increased expression of this receptor or to a shift in the affinity resulting from the chronic exposure to a higher pericellular ATP level. We were unable to show appreciable difference in P2X<sub>7</sub> expression between T2D and control fibroblasts. On the contrary, the B<sub>2</sub>ATP dose-dependency curve was shifted leftward in T2D fibroblasts, and ATP exhibited an increased potency at the T2D P2X<sub>7</sub>. Thus, we believe that the higher sensitivity of T2D fibroblasts to ATP is attributable to a change in intrinsic receptor properties rather than to a change in expression. We presently do not know the molecular basis for such an increased sensitivity. We speculated initially that it might be caused by a priming effect dependent on the previous exposure to high ATP concentrations, as described in microglial cells, but experiments shown in Figure IIC show that the extracellular ATP level typical of T2D fibroblasts causes desensitization rather than priming.

Our data thus suggest that an enhanced sensitivity to ATP of the P2X<sub>7</sub> receptor and a higher basal rate of ATP release might be primary dysfunctions affecting P2 receptor signaling in T2D fibroblasts. Although the increased fibronectin deposition in the diabetic arterial wall is well documented, little is known about the role that apoptosis may play in the pathogenesis of diabetic angiopathy. Few reports to date attempted to establish a correlation between increased apoptosis and accelerated atherosclerosis in diabetic patients. Crucial events underlying the most serious clinical outcomes, such as plaque erosion, rupture, and occlusive thrombi development, are still only partially understood. We think the demonstration that T2D fibroblasts have an intrinsic alteration in P2 receptor signaling unveils an interesting and as yet unfathomed mechanism that on one hand alters the cellular and extracellular structural components of the arterial wall, and on the other hand, generates a proinflammatory milieu. Either event is crucial in the pathogenesis of vascular damage in diabetes, thus we anticipate that a deeper understanding of the physiology of the P2 receptor system in diabetes will also lead to the development of novel therapeutic approaches.

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Methods

Cell culture

Skin biopsies were performed on the forearm surface of five control subjects and five T2D patients (Table I). Specimens were cut into small pieces, washed in culture medium and placed at the bottom of a 25-cm² plastic culture flasks in a CO₂ incubator at 37° C. After 15 minutes, 5 ml of medium were added. The biopsies were then supplemented with DMEM (Sigma, Munich, Germany) containing 10% FCS, L-glutamine (4 mM) and 100 U/ml penicillin (Bristol-Myers Squibb, New York, NY, USA) and 100 µg/ml streptomycin (Sigma). At confluence, fibroblasts were washed twice with phosphate-buffered saline and treated with a 1:5 trypsin- solution (in PBS) for 3 min at 37° C. The cell suspension was then centrifuged, the pellet resuspended in DMEM and transferred to a 80-cm² plastic flask. Fibroblasts from healthy or T2D subjects were grown in DMEM containing physiological (5 mM) glucose concentration. All experiments were performed between the fourth and the tenth passage.

Cell permeability assay

Cell permeabilization was assessed by monitoring YO-PRO (Molecular Probes, Leiden, The Netherlands) or lucifer yellow (LY) (Molecule Probes) uptake [27, 28]. Cells monolayers (10⁵ cells/well) were incubated at 37° C in the presence of 10 µM YO-PRO or 1 mg/ml LY, with either 2 mM ATP or benzoyl ATP (BzATP). Controls were incubated in the absence of nucleotides. After 60-90 min, cell were rinsed twice with PBS at room temperature, and samples immediately examined with an inverted fluorescence microscope (Olimpus IMT-2; Olimpus Optical Co, Ldt., Tokio, Japan) equipped with a 40x objective and a fluorescein filter.

Evaluation of apoptosis

Apoptosis was assessed by measuring nucleosome formation (Cell Death Detection kit, Boheringer Mannheim, Mannheim, Germany), and nuclear condensation following cell treatment with ATP or
other nucleotides for 3-5 hours. Nuclear condensation was evaluated by staining nucleotide-treated
and control cells with ethidium bromide at a concentration of 100 µM.

**Immunofluorescence**

Cells (10^6/ml) were seeded on glass coverslips, rinsed with PBS and fixed with paraformaldehyde
(2% in PBS). After 2 hours, they were permeabilized with Triton X-100 (1% in PBS) and incubated
for 1 h with the anti-AP-1 monoclonal antibody (1:40 dilution in PBS) (29). Cell were then rinsed
three times with PBS and incubated for 30 minutes with an anti-mouse Ig FITC-labelled antibody
(1:50 dilution in PBS). At the end of this incubation, coverslips were rinsed 3 times and analysed
with a TE-300 Nikon (Nikon Co., Tokio, Japan) fluorescence microscope. In order to allow a
comparison of fluorescence intensity of the different cell samples, exposures times of fluorescence
pictures shown in Figures 1 and 2 were exactly the same.

**IL-6 assay**

To induce IL-6 secretion, fibroblasts (10^5/ml) were treated with lipopolysaccharide (LPS) and
phorbol myristate acetate (PMA) prior to nucleotide addition. The cell-free supernatants from
fibroblasts cultures were collected at the end of a 1 h incubation with increasing doses of ATP, and
IL-6 was determined using commercial enzyme-linked immunosorbent (ELISA) kits (Immuno
Pharmacology Research, Catania, Italy). All samples and standards were assayed in triplicate.

**Determination of extracellular ATP**

Fibroblasts were seeded at a concentration of 10^5 cells/well in microtiter plastic dishes in a total
volume of culture medium of 100 l, rinsed and supplemented with 100 l of diluent buffer
(FireZyme, San Diego, CA) to stabilize extracellular ATP, and placed directly into the test chamber
of a luminometer (FireZyme). Then, 100 l of a luciferin-luciferase solution (FireZyme) was
added, and light emission was recorded.
**Plasma membrane potential measurements**

Changes in plasma membrane potential were measured with the fluorescent dye bis[1,3-diethylthiobarbiturate] trimethineoxonal (bisoxonol) (Molecular Probes) at the wavelength pair 540/580 nm. Experiments were performed in cell suspensions (10^5 fibroblasts/ml) in a Perkin Elmer spectrofluorimeter equipped with a thermostat-controlled (37°C) cuvette holder and magnetic stirring.

**Western blotting**

Fibroblast (106/ml) were lysed in lysis buffer containing 300 mM sucrose, 1 mM K$_2$HPO$_4$, 1 mM MgSO$_4$, 5.5 mM glucose, 20 mM Heps, pH7.4, 1 mM benzamidine, 1 mM PMSF, 0.2 μg of Dnase and 0.2 μg of RNase by three cycles of freeze/thawing. Proteins (30 μg in each lane) were separated on 7.5% SDS polyacrylamide gel, and blotted on a sheet of nitrocellulose paper incubated with the rabbit polyclonal anti-P2X$_7$ serum.

**FACS analysis**

Plasma membrane expression of the P2X$_7$R was measured with a single ion argon laser (FACScan, Becton Dickinson, San José, CA USA) cytometer. The P2X$_7$R was analyzed with indirect immunofluorescence using a primary anti- P2X$_7$ monoclonal antibody raised in our laboratory and an anti-mouse FITC-labelled goat monoclonal antibody. Individual cell samples were incubated for 30 min with the primary and further 30 min with the secondary antibody. To further validate analysis of P2X$_7$R expression, fibroblasts were also analyzed for expression of the known surface marker MHC class I (MHC I) with the mouse monoclonal antibody W6/32 (ATCC, Rockville MD, USA). Null samples were run by using nonreactive isotypic antibody purchased from Becton Dickinson. Ten thousand events were acquired for each condition and analyzed with the computer software LYSYS II (Becton Dickinson).
TABLE I

Clinical parameters of control and T2D subjects

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<th>Controls</th>
<th>T2D</th>
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<tr>
<td>Age (yrs)</td>
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<td>48±5</td>
</tr>
<tr>
<td>Gender (M/F)</td>
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<td>3/2</td>
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<tr>
<td>Body Mass Index (Kg/m²)</td>
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<td>28.2±1.7</td>
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<td>Diabetes duration (yrs)</td>
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<td>Fasting plasma glucose (mmol/l)</td>
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<td>7.8±0.5*</td>
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<tr>
<td>HbA1c (%)</td>
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<tr>
<td>Diastolic blood pressure</td>
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* $p \leq 0.001$ vs controls by Student’s t test
Figure Legends

**Figure I**  P2X₇ pore formation in T2D and control fibroblasts.

Fibroblast monolayers from T2D (panels A, B, E and F) or control (panels C, D, G, H) subjects were incubated in DMEM in the presence of YO-PRO with (panels E-F) or without (panels A-D) ATP as described in Materials and Methods. Panels A, C, E, G, fluorescence; panels B, D, F, H, phase contrast. Magnification 40X. Bar=25 µm.

**Figure II**  P2X₇ receptor expression and affinity in T2D and control fibroblasts; expression of P2X₇ protein. Fibroblast monolayers were processed for immunoblotting as described in Materials and Methods. Immunostaining of the P2X₇ receptor protein solubilized from the mouse microglial line N13 is shown for comparison. Lower graph, densitometry.

**Figure III**  FACS analysis of P2X₇ expression in control and T2D fibroblasts. See Materials and Methods for details.

**Figure IV**  Nucleotide-induced plasma membrane depolarization. Monolayers were detached by trypsinization (1:5 in PBS), rinsed three times and suspended in saline solution (see Materials and Methods). Depolarization is expressed as percentage of maximal plasma membrane collapse caused by 60 mM KCl. Panels a and b, squares, healthy subjects; circles, T2D patients. In panels c and d, T2D fibroblasts were preincubated for 30 min in the presence (circles) or absence (squares) of apyrase (1 U/ml) before stimulaton with the nucleotides. Data are averages±SD of triplicate determinations from a single experiments repeated three times for each of subjects studied.
Figure 1
Figure II
Figure III
Figure IV