Enhanced $P2X_7$ 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 $P2X_7$ receptor in fibroblasts from healthy subjects and patients with type 2 diabetes.

Methods and Results—Fibroblasts were isolated from skin biopsies. $P2X_7$ 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 $P2X_7$-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 $P2X_7$ 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.[1,2] 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.[3,4] 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.[5,6] 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.[7] In turn, activated fibroblasts proliferate and migrate into the plaque, contributing to plaque thickening and fibrous cap formation.[8] In the atheromatous 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.[8] 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.[9]

Receptors for extracellular nucleotides (P2 receptors) are a focus of increasing attention in vascular biology and pathology.[10,11] Extracellular ATP is known to cause vasodilation, probably mediated by NO release,[12] or alternatively, contraction, probably mediated by direct smooth muscle cell stimulation.[13] In addition, ATP induces cytokine secretion,[14] chemotaxis of inflammatory cells,[15] smooth muscle cell proliferation,[16] or cytotoxicity.[17] Among other nucleotides, ADP has a very important role in vascular biology for its powerful platelet-aggregating action.[18]

Effects of extracellular nucleotides are mediated via activation of 2 families of distinct cell surface receptors, P2X and P2Y.[19] We recently investigated expression and function of

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From the Department of Internal Medicine (A.S.), University of Pisa, Italy; the Department of Experimental and Diagnostic Medicine (P.C., A.M., E.A., R.R., O.R.B., F.D.V.), University of Ferrara, Italy; and the Interdisciplinary Center for the Study of Inflammation (F.D.V.), University of Ferrara, Italy.

Reprint requests to Dr Francesco Di Virgilio, Department of Experimental and Diagnostic Medicine, Section of General Pathology, University of Ferrara, Via Borsari 46, I-44100 Ferrara, Italy. E-mail fdv@unife.it

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the P2X$_7$ receptor in human fibroblasts and have discovered that P2X$_7$-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.[20] The P2X$_7$ 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[23,24] and its ability to mediate massive release of interleukin 1β (IL-1β).[25,26] In the present work, we extended the investigation of P2X$_7$-mediated responses to fibroblasts from type 2 diabetes (T2D) patients. Our data show that P2X$_7$-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$_7$ 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.[21,27,28] Figure I (available online at http://atvb.ahajournals.org) shows fluorescence microscopy 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$\pm$SD) 68.42$\pm$28.30 (n=35) and 159.80$\pm$39.15 (n=65) for fibroblasts from healthy and T2D subjects, respectively ($P<0.001$; Student t test or ANOVA). Similar results were obtained at a lower concentration (0.5 mmol/L) of the more potent P2X$_7$ 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$_7$ 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.[20,29] 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 1E and 1F). 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

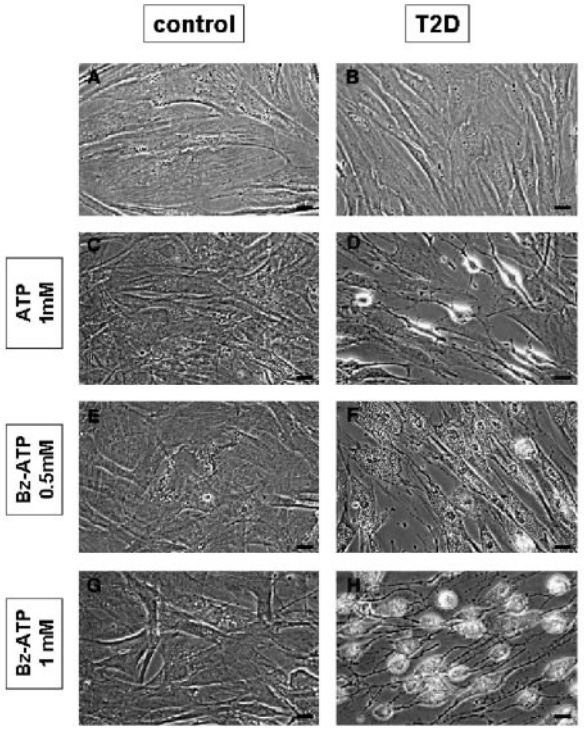

Figure 1. Nucleotide-dependent shape changes and microvesicle formation in T2D and control fibroblasts. Fibroblast monolayers from control (A, C, E, and G) and T2D subjects (B, D, F, and H) were incubated at 37°C in DMEM for 2 hours in the presence of 1 mmol/L ATP (C, D), 0.5 mmol/L BzATP (E, F), 1 mmol/L BzATP (G, H), or left untreated (A, B). At the end of the incubation time, monolayers were rinsed and viewed with a $\times$40 objective. Bar=25 μm.

microvesicles and that the average number of microvesicles per cell$\pm$SD was 75$\pm$25 (n=85). T2D cells that did not contain microvesicles were frankly apoptotic. Very few cells from healthy controls (<5%) underwent microvesiculation, and microvesicle content was 10$\pm$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.[17,21,30] 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.[31] 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$\pm$41.50 and 152.53$\pm$27.30 for healthy (n=45) and T2D (n=73) fibroblasts, respectively ($P<0.01$). Enhanced intracellular accu-

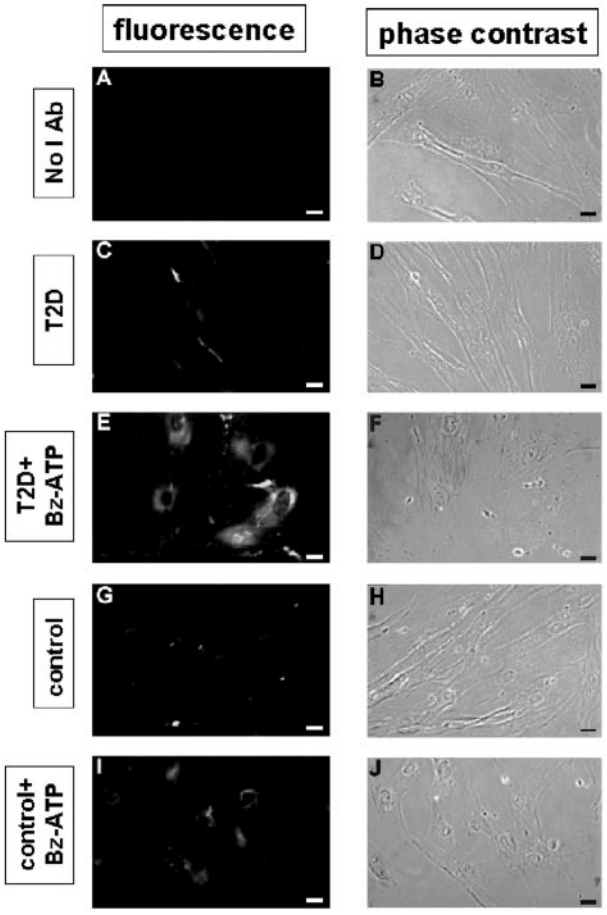

Figure 2. Cytoplasmic microvesicles contain fibronectin. Fibroblast monolayers from control (A, B, and G through J) or T2D (C through F) subjects were incubated in DMEM with (E, F, I, and J) or without (A through D, G, and H) 1 mmol/L BzATP for 1 hour at 37°C, fixed, and processed for immunofluorescence as described in Materials and Methods. In A and B, no primary antibody (Ab) was added. Bar=25 μm.

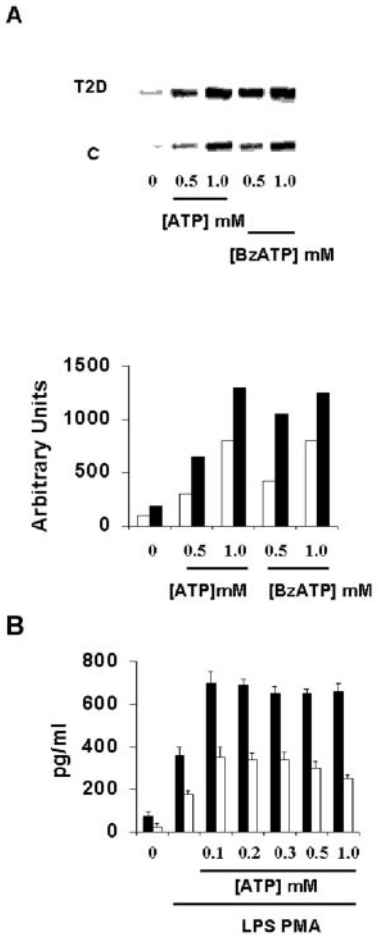

Figure 3. Increased ATP-stimulated release of fibronectin and IL-6 from T2D fibroblasts. A, Fibroblast monolayers in DMEM were stimulated with the indicated nucleotide concentrations for 1 hour at 37°C. At the end of this incubation, supernatants were withdrawn and assayed for fibronectin content by immunoblotting. Bottom of A shows a densitometric analysis of the bands. B, Fibroblast monolayers in DMEM were preincubated for 2 hours with LPS (1 μg/mL) and PMA (100 nM), then they were treated with increasing nucleotide concentrations for 1 hour. Supernatants were withdrawn and assayed for IL-6 content by ELISA. Data for IL-6 release are averages±SD of triplicate determinations from a single experiment. Each experiment was repeated 3× for each control or T2D subject. Cell supernatants were checked routinely for lactic dehydrogenase content that was always <10% whether in the absence or presence of the nucleotides. Closed bars represent T2D; open bars, healthy subjects.

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).[20] 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 $P2X_7$ 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,

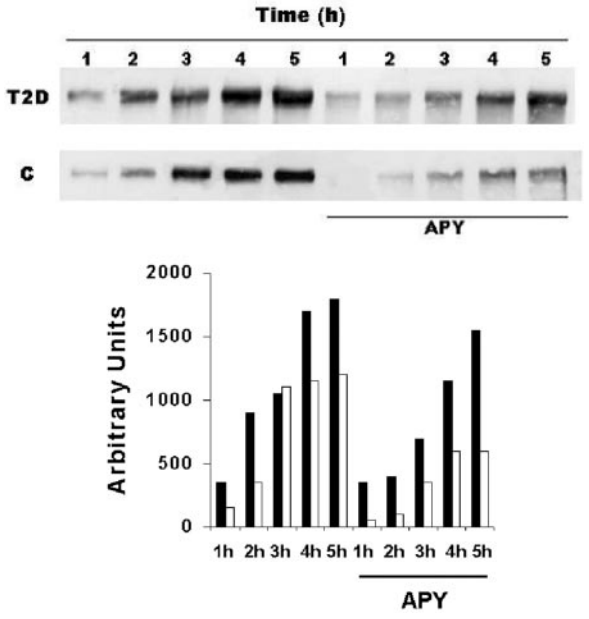

Figure 4. Apyrase reduces basal fibronectin release. Fibroblast monolayers were incubated in DMEM in the absence or presence of apyrase (Apy; 0.4 U/mL). No exogenous nucleotides were added. At the end of this incubation, supernatants were withdrawn and assayed for fibronectin content by immunoblotting. Bottom, Densitometric analysis. Data are from 1 experiment representative of 3 others. Closed bars represent T2D; open bars, healthy subjects.

apyrase largely reduced basal secretion of fibronectin from T2D and control fibroblasts, thus supporting a central role of the ATP-based autocrine–paracrine loop.

P2X$_7$ Expression

Enhanced susceptibility of T2D fibroblasts to ATP-mediated cytotoxicity suggested that P2X$_7$ might be expressed at a higher level in these cells. However, immunoblot analysis with a polyclonal antibody raised against the COOH tail of P2X$_7$ failed to show an enhanced staining (also see densitometry) of T2D compared with control fibroblasts (Figure II, available online at http://atvb.ahajournals.org). Fluorescence-activated cell sorter analysis of control and T2D fibroblasts was performed to compare surface P2X$_7$ expression. As shown in Figure III (available online at http://atvb.ahajournals.org), staining with a P2X$_7$-specific monoclonal antibody revealed that the healthy cells were rather dishomogeneous, with various levels of P2X$_7$ surface expression. On the contrary, the T2D fibroblasts were much more homogenous, showing basically only 1 population. We also stained the fibroblasts for a surface marker unrelated to P2X$_7$, the class I major histocompatibility complex antigen, which showed a similar fluorescence pattern. The percentage of P2X$_7$-positive cells was very similar and mean fluorescence intensity of healthy compared with T2D fibroblasts was not statistically different (13.65±2.55 versus 14.13±3.56 FU for healthy versus T2D fibroblasts, respectively). Finally, we performed an ATP dose dependency of P2X$_7$ activation. As a readout, we choose plasma membrane depolarization, which is one of the earliest responses induced by opening the P2X$_7$ pore. Figure IV (available online at http://atvb.ahajournals.org) 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, P2X$_7$ 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 P2X$_7$-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 hydrophylic solutes (eg, lucifer yellow or YO-PRO) that is gener-

ally considered the hallmark of $P2X_7$ function is usually delayed and of low intensity.[29] 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,[29] we assigned these vesicules 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.[31,44] 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 $P2X_7$ 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 $P2X_7$ 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 $P2X_7$ 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 $P2X_7$ expression between T2D and control fibroblasts. On the contrary, the BzATP dose-dependency curve was shifted leftward in T2D fibroblasts, and ATP exhibited an increased potency at the T2D $P2X_7$. 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,[45] but experiments shown in Figure IIC show that the high extracellular ATP level typical of T2D fibroblasts causes desensitization rather than priming.

Our data thus suggest that an enhanced sensitivity to ATP of the $P2X_7$ 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.[46,47] 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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References

1. Wilcox JN, Scott NA. Potential role of the adventitia in arteritis and atherosclerosis. *Int J Cardiol*. 1996;54:S21–S35.
2. Strauss BH, Rabinovitch M. Adventitial fibroblasts: defining a role in vessel wall remodeling. *Am J Respir Cell Mol Biol*. 2000;22:1–3.
3. Buckley CD, Pilling D, Lord JM, Akbar AN, Scheel-Toellner D, Salmon M. Fibroblasts regulate the switch from acute resolving to chronic persistent inflammation. *Trends Immunol*. 2001;22:199–204.
4. Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C, Brown RA. Myofibroblasts and mechano-regulation of connective tissue remodeling. *Nat Rev Mol Cell Biol*. 2002;3:349–363.
5. Strehlow D, Korn JH. Biology of scleroderma fibroblast. *Curr Opin Rheumatol*. 1998;10:572–578.
6. Mansbridge JN, Liu K, Pinney RE, Patch R, Ratcliffe A, Naughton GK. Growth factors secreted by fibroblasts: role in healing diabetic foot ulcers. *Diabetes Obes Metab*. 1999;1:265–279.
7. Sartore S, Chiavegato A, Faggin E, Franch R, Puato M, Ausoni S, Pauletto P. Contribution of adventitial fibroblasts to neointima formation and vascular remodeling: from innocent bystander to active participant. *Circ Res*. 2001;89:1111–1121.
8. Wilcox JN, Scott NA. Potential role of the adventitia in arteritis and atherosclerosis. *Int J Cardiol*. 1996;54:S21–S35.
9. Kolodgie FD, Burke AP, Farb A, Gold HK, Yuan J, Narula J, Finn AV, Virmani R. The thin-cap fibroatheroma: a type of vulnerable plaque, the major precursor lesion to acute coronary syndromes. *Curr Opin Cardiol*. 2001;16:285–292.
10. Boarder MR, Hourani SM. The regulation of vascular function by P2 receptors: multiple sites and multiple receptors. *Trends Pharmacol Sci*. 1998;19:99–107.
11. Bunstock G. Purinergic signaling and vascular cell proliferation and death. *Arterioscler Thromb Vasc Biol*. 2002;22:364–373.
12. You J, Johnson TD, Marrelli SP, Mombouli JV, Bryan RM Jr. P2U receptor-mediated release of endothelium-derived relaxing factor/nitric oxide and endothelium-derived hyperpolarizing factor from cerebrovascular endothelium in rats. *Stroke*. 1999;30:1125–1133.
13. Boland B, Himpens B, Vincent MF, Gillis JM, Casteels R. ATP activates P2X-contracting and P2Y-relaxing purinoceptors in the smooth muscle of mouse vas deferens. *Br J Pharmacol*. 1992;107:1152–1158.
14. Griffiths RJ, Stam EJ, Downs JT, Otterness IG. ATP induces the release of IL-1 from LPS-primed cells in vivo. *J Immunol*. 1995;154:2821–2828.

15. Chaulet H, Desrganges C, Renault MA, Dupuch F, Ezan G, Peiretti F, Loirand G, Pacaud P, Gadeau AP. Extracellular nucleotides induce arterial smooth muscle migration via osteopontin. *Circ Res*. 2001;89: 772–778.
16. Wang DJ, Huang NN, Heppel LA. Extracellular ATP and ADP stimulate proliferation of porcine aortic smooth muscle cells. *J Cell Physiol*. 1992; 153:221–233.
17. Di Virgilio F, Bronte V, Collavo D, Zanovello P. Responses of mouse lymphocytes to extracellular adenosine 5′-triphosphate (ATP). Lymphocytes with cytotoxic activity are resistant to the permeabilizing effects of ATP. *J Immunol*. 1999;143:1955–1960.
18. Burnstock G, Williams M. P2 purinergic receptors: modulation of cell function and therapeutic potential. *J Pharmacol Exp Ther*. 2000;295: 862–969.
19. Ralevic V, Burnstock G. Receptors for purines and pyrimidines. *Pharmacol Rev*. 1988;50:413–492.
20. Solini A, Chiozzi P, Falzoni S, Morelli A, Fellin R, Di Virgilio F. High glucose modulates P2X$_7$ receptor-mediated function in human primary fibroblasts. *Diabetologia*. 2000;43:1248–1256.
21. Steinberg TH, Silverstein SC. Extracellular ATP4 promotes cation fluxes in the J774 mouse macrophage cell line. *J Biol Chem*. 1997;262: 3118–3122.
22. Steinberg TH, Newman AS, Swanson JA, Silverstein SC. ATP4 permeabilizes the plasma membrane of mouse macrophages to fluorescent dyes. *J Biol Chem*. 1987;262:8884–8888.
23. Di Virgilio F. The P2Z purinoceptor: an intriguing role in immunità, inflammation and cell death. *Immunol Today*. 1995;16:524–528.
24. Surprenant A, Rassendren F, Kawashima E, North RA, Buell G. The cytolytic P2Z receptor for extracellular ATP identified as a P2X receptor (P2X$_7$). *Science*. 1996;272:735–738.
25. Ferrari D, Chiozzi P, Falzoni S, Dal Susino M, Melchiorri L, Baricordi OR, Di Virgilio F. Extracellular ATP triggers IL-1β release by activating the purinergic P2Z receptor of human macrophages. *J Immunol*. 1997; 159:1451–1458.
26. Solle M, Labasi J, Perregaux DG, Stam E, Petrushkova N, Koller BH, Griffiths RJ, Gabel CA. Altered cytokine production in mice lacking P2X$_7$ receptors. *J Biol Chem*. 2001;276:125–132.
27. Falzoni S, Munerati M, Ferrari D, Spisani S, Moretti S, Di Virgilio F. The purinegic receptor of human macrophage cells, Characterization and possible physiological role. *J Clin Invest*. 1995;95:1207–1216.
28. Virginio C, Church D, North RA, Surprenant A. Effects of divalent cations, protons and calmidazolium at the rat P2X$_7$ receptor. *Neuropharmacology*. 1997;36:1285–1294.
29. Solini A, Chiozzi P, Morelli A, Fellin R, Di Virgilio F. Human primary fibroblasts in vitro express a purinergic P2X$_7$ receptor coupled to ion fluxes, microvesicle formation and IL-6 release. *J Cell Sci*. 1999;112:297–305.
30. Virginio C, MacKenzie A, North RA, Surprenant A. Kinetics of cell lysis, dye uptake and permeability changes in cells expressing the rat P2X$_7$ receptor. *J Physiol*. 1999;519:335–346.
31. Rasmussen LM, Heickendorff L. Accumulation of fibronectin in aortas from diabetic patients. A quantitative immunohistochemical and biochemical study. *Lab Invest*. 1989;61:440–446.
32. Shats EA, Nair CH, Dhall DP. Interaction of endothelial cells and fibroblasts with modified fibrin networks: role in atherosclerosis. *Atherosclerosis*. 1997;29:9–15.
33. Duner E, Di Virgilio F, Trevisan R, Cipollina MR, Crepaldi G, Nosadini R. Intracellular free calcium abnormalities in fibroblasts from non-insulin-dependent diabetic patients with and without hypertension. *Hypertension*. 1997;29:1007–1013.
34. Chiquette E, Chilton R. Cardiovascular disease: much more aggressive in patients with type 2 diabetes. *Curr Atheroscler Rep*. 2002;4:134–142.
35. Lopes-Virella MF, Virella G. Cytokines, modified lipoproteins and arteriosclerosis in diabetes. *Diabetes*. 1996;45(suppl 3):S40–S44.
36. Loot MA, Kenter SB, Au FL, van Galen WJ, Middelkoop E, Bos JD, Mekkes JR. Fibroblasts derived from chronic diabetic ulcers differ in their response to stimulation with EGF, IGF-I, bFGF and PDGF-AB compared to controls. *Eur J Cell Biol*. 2002;81:153–160.
37. Twigg SM, Chen MM, Joly AH, Chakrapani SD, Tsubaki J, Kim HS, Oh Y, Rosenfeld RG. Advanced glycosylation end products up-regulate connective tissue growth factor (insulin-like growth factor-binding protein related protein 2) in human fibroblasts: a potential mechanism for expansion of extracellular matrix in diabetes mellitus. *Endocrinology*. 2001;142:1760–1769.
38. Bodin P, Burnstock G. Increased release of ATP from endothelial cells during inflammation. *Inflamm Res*. 1998;47:351–354.
39. Mizumoto N, Kumamoto T, Robson SC, Sevigny J, Matsue H, Enjyoji K, Takashima A. CD39 is the dominant Langerhans cell-associated ecto-NTDPase: modulatory roles in inflammation and immune responsiveness. *Nat Med*. 2002;8:358–365.
40. Burnstock G. Potential therapeutic targets in the rapidly expanding field of purinergic signaling. *Clin Med*. 2002;2:45–53.
41. Dubyak GR. Focus on "extracellular ATP signaling and P2X nucleotide receptors in monolayers of primary human vascular endothelial cells." *Am J Physiol Cell Physiol*. 2002;282:C242–C244.
42. Ward PA. Mechanism of endothelial cell killing by H_2O_2 or products of activated neutrophils. *Am J Med*. 1991;91:89S–94S.
43. Di Virgilio F, Solini A. P2 receptors: new potential players in atherosclerosis. *Br J Pharmacol*. 2002;135:831–842.
44. Andresen JL, Rasmussen LM, Ledet T. Diabetic macroangiopathy and atherosclerosis. *Diabetes*. 1996;45:S91–S94.
45. Chakfe Y, Seguin R, Antel JP, Morissette C, Malo D, Henderson D, Seguela P. ADP and AMP induce interleukin-1β release from microglial cell through activation of ATP-primed P2X$_7$ receptor channels. *J Neurosci*. 2002;22:3061–3069.
46. Schneider DB, Vassalli G, Wen S, Driscoll RM, Sassani AB, De Young MB, Linnemann R, Virmani R, Dichek DA. Expression of Fas ligand in arteries of hypercholesterolemic rabbits accelerates atherosclerotic lesion formation. *Arterioscl Thromb Vasc Biol*. 2000;20:298–308.
47. Guevara NV, Kim HS, Antonova EI, Chan L. The absence of p53 accelerates atherosclerosis by increasing cell proliferation in vivo. *Nat Med*. 1999;5:335–339.