High glucose modulates P2X₇ receptor-mediated function in human primary fibroblasts

A. Solini¹, P. Chiozzi², S. Falzoni², A. Morelli², R. Fellin¹, F. Di Virgilio²

¹ Department of Clinical and Experimental Medicine, Section of Internal Medicine, University of Ferrara, Ferrara, Italy

² Department of Experimental and Diagnostic Medicine, Section of General Pathology, University of Ferrara, Ferrara, Italy

Abstract

Aims/hypothesis. Purinergic receptors are a family of newly characterized plasma membrane molecules involved in several and as yet only partially known cellular functions such as vascular reactivity, apoptosis and cytokine secretion. Little is known about the effect extracellular microenvironment has on their function. Fibroblasts share several features with smooth muscle cells and are an important constituent of the atherosclerotic plaque. Our aim was to evaluate the effect of high glucose concentration on ATP-mediated responses in human fibroblasts.

Methods. Fibroblasts were obtained by skin biopsies and grown at two different glucose concentrations. We evaluated receptor expression by RT-PCR and immunoblotting and receptor localization by immunofluorescence. Plasma membrane potential and calcium changes were measured by fluorescent indicators. Apoptosis was determined by ethidium bromide staining and caspase-3 activation.

Results. We show that cells grown in a medium with high glucose concentration underwent great ATP-mediated morphological changes, enhanced apoptosis, caspase 3 activation and interleukin-6 release. We identified P2X₇ as the main purinergic receptor involved in these responses. Furthermore, high glucose concentration triggered the assembly of P2X₇ into ring-like structures located at the periphery of the cells.

Conclusion/interpretation. Given that ATP is frequently released into the extracellular milieu upon cell and tissue damage, secretory exocytosis or activation of plasma membrane transporters, we hypothesize that ATP receptors participate in the pathogenesis of vascular complications of diabetes. [Diabetologia (2000) 43: 1248–1256]

Keywords Calcium, interleukin-6, P2 purinergic receptor, apoptosis, hyperglycaemia.

Extracellular ATP triggers a variety of responses in several cell types, including contraction of smooth muscle cells [1], regulation of nitric oxide production from endothelium [2], stimulation of cytokine release from immune cells [3, 4], and modulation of several

other metabolic pathways [5, 6]. Moreover, it is a strong stimulus for insulin release from isolated pancreas and beta cells [7, 8], which points to a potentially important role for this nucleotide in the control of blood glucose. Recently, ATP receptors have also been found to be involved in the regulation of GLUT-4 expression in cardiomyocytes [9].

The receptors mediating these diverse effects have been described as P2 purinoreceptors and are divided into two subgroups: P2Y and P2X receptors [10]. The P2Y are seven membrane spanning, and G protein-coupled receptors, linked to inositol phosphate and diacylglycerol formation. They are nearly ubiquitous, have a high affinity for ATP (K_m in the nano-micro

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Corresponding author: Dr. F. Di Virgilio, Department of Experimental and Diagnostic Medicine, Section of General Pathology, University of Ferrara, Via Borsari, 46, I-44100 Ferrara, Italy

Abbreviations: CTP, cytidine triphosphate; BzATP, Benzoyl ATP; $[Ca^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; PMA, phorbol myristate acetate; LPS, lipopolysaccharide.

molar range) and, depending on the subtype, are activated by various nucleotides [ATP, ADP, uridine triphosphate (UTP), GTP] [11]. The P2X are a family of multimeric ligand-gated ion channels solely activated by extracellular ATP and structurally distinct from other ligand-gated channels such as the nicotinic or γ -amino-*n*-butyric acid (GABA) receptors. Seven different P2X subunits (P2X₁ to P2X₇) have been cloned and sequenced [12]. Among P2X receptors, P2X₇ (previously known as P2Z) has attracted much interest as it has been shown to be involved in cell death, shedding of surface antigens and cytokine release [13–15]. Moreover, recent data suggest that this receptor also mediates activation of the transcription factors nuclear factor κ B (NF- κ B) and nuclear factor of activated T cells (NFAT) [16, 17].

We have previously shown that human fibroblasts express P2X₇ and that stimulation of these cells by high concentrations of ATP causes pronounced microvesiculation and release of the proinflammatory cytokine IL-6 [18].

It is increasingly appreciated that fibroblasts and their secretory products play a central part in the pathogenesis of atherosclerotic plaque and microvascular complications of diabetes [19, 20]. In this study we investigated the effect of high glucose concentration on fibroblast responses to ATP, in the light of the hypothesis that the diabetic microenvironment sensitizes fibroblasts to nucleotide stimulation, thus exacerbating tissue damage.

Materials and methods

Cells. Human fibroblasts were obtained from a skin biopsy on the anterior surface of the forearm in four healthy volunteers. Fibroblasts were cultured in DMEM, supplemented with 10% FCS at two different D-glucose concentrations (5.5 and 22 mmol/l) at 37°C. Immediately after biopsy, fibroblasts were split into two batches and grown in the presence of 5.5 or 22 mmol/l D-glucose. To exclude the possible role of hypertonicity, mannitol was systematically added to medium containing 5.5 D-glucose to bring total osmolarity to a value equivalent to that of the medium containing 22 mmol/l D-glucose. No difference in responses was observed between cultures grown in the presence of 16.5 mmol/l mannitol or 5.5 mmol/l glucose. After the fourth passage, cells were harvested and stored in liquid nitrogen. For each experiment, fibroblasts were thawed and grown as described above. All experiments were done in cells made quiescent after the sixth passage.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from fibroblasts as described previously [21]. Reverse transcription was carried out using the following specific primers: sense, 5'-AGA TCG TGG AGA ATG GAG TG-3'; antisense, 3'-TTC TCG TGG TGT AGT TGT GG-3'; the probe was 5'-TCA TGC ACT ACA CAC CTT CC-3'. Oligonucleotides were synthesized by M-Medical Genenco-Life Science (Firenze, Italy). Labelling of the probe and blotting were carried out as described in Dig-labelling and detection protocols (Boehringer Mannheim, Germany). The RT-PCR products were fractionated in 1.2% agarose gel

and transferred to a positively charged nylon membrane (ICN Biomedicals Aurora, Ohio) by a vacuum blotting system (Bio-Rad Laboratories, Hercules, Calif., USA) for 2 h. Hybridization signals were analysed by detection of chemiluminescence after incubation with a dilution of anti-digoxigenin Fab conjugated to alkaline phosphatase.

Immunoblotting. Cells were lysed in a lysis buffer containing 300 mmol/l sucrose, 1 mmol/l K₂HPO₄, 1 mmol/l MgSO₄, 5.5 mmol/l glucose, 20 mmol/l HEPES, 1 mmol/l benzamidine, 1 mmol/l PMSF, 0.2 mg DNase and 0.2 mg RNase by repeated freeze/thawing (three cycles). Proteins were separated on 7.5% SDS polyacrylamide gel [22], and blotted overnight on nitrocellulose paper. The rabbit polyclonal anti-P2Z/P2X₇ serum was kindly provided by Dr G. Buell (SeroPharmaceutical Research Institute-Geneva, Switzerland) and was used at a dilution of 1:100 in Tween 20 TRIS-buffered saline (TBST) buffer (10 mmol/l TRIS-Cl, 150 mmol/l NaCl, 0.1% Tween 20, pH 8.0). Secondary Ab was a goat anti-rabbit Ab conjugated to alkaline phosphatase.

Immunofluorescence. For immunofluorescence fibroblasts were plated on glass coverslips, rinsed with PBS and fixed with 2% paraformaldehyde in PBS. Fixed cells were permeabilized with Triton X-100 (30 min, 0.1% in PBS), incubated for 20 min in FCS (2% in PBS), rinsed and incubated at 4°C overnight with the primary antibody (at a 1:50 dilution). The secondary antibody [fluorescein isothiocyanate (FITC)-labelled anti-rabbit] was added at a 1:200 dilution for 30 min. Blocking experiments with the peptide used for immunization were done to check selectivity of the primary antibody. Cells were then thoroughly rinsed and observed with either a IMT-2 Olympus (Olympus Optical Co., Tokyo, Japan) or a TE-300 Nikon (Nikon Co., Tokyo, Japan) fluorescence microscope.

Caspase activation. Caspase 3 activation was measured fluorimetrically with the EnzChek Caspase-3 Assay Kit (Molecular Probes, Eugene, Ore., USA) as indicated by the manufacturer. Enzyme activity was measured as increase in fluorescence emission at 530 ± 5 nm, using excitation at 485 ± 5 nm, at room temperature. The fluorescence increase given by 100 µl of supernatant from 2 × 10⁵ fibroblasts incubated 3 h with 5 mmol/l ATP at 37°C corresponded to 100 Arbitrary Units.

Interleukin-6 secretion. Interleukin-6 was measured with the BioSource Cytoscreen Immunoassay kit (BioSource International Camarillo, Calif., USA).

Measurement of plasma membrane potential. Changes in plasma membrane potential were measured with the fluorescent dye bis-oxonol at the wavelength pair 540 to 580 nm, as described previously [18]. Unless otherwise stated, all experiments were done at 37°C in saline medium containing: 125 mmol/l NaCl, 5 mmol/l KCl, 1 mmol/l MgSO₄, 1 mmol/l Na₂HPO₄, 5.5 mmol/l glucose, 5 mmol/l NaHCO₃ and 20 mmol/l HEPES, in the absence or presence of Ca²⁺ 1 mmol/l. In the absence of Ca²⁺, EGTA 0.5 mmol/l was added. All fluorescence measurements were done in a Perkin-Elmer LS50 spectrofluorimeter (Perkin-Elmer, Norwalk, Conn., USA) equipped with a thermostatically controlled cuvette holder and a magnetic stirrer.

Cytoplasmic free Ca²⁺ ([Ca²⁺]_i) fluorimetry. Cells were loaded with the fluorescent indicator Fura 2/AM (Boehringer Mannheim Biochimica, Germany), at a concentration of 2 µmol/l for 30 min at 37°C in a saline solution containing Ca²⁺. After loading, cells were rinsed and the coverslips were placed in a

thermostatically controlled (37°C) dual-wavelength spectrofluorimeter (Perkin-Elmer LS50), equipped with a quartz cuvette holder. Measurement of fluorescence was obtained with alternating excitation wavelengths of 340 and 380 nm and an emission wavelength of 505 nm. We evaluated $[Ca^{2+}]_i$ in the resting condition and after rapid injection of increasing concentrations of nucleotides [ATP, Cytidine triphosphate (CTP), benzoyl ATP (BzATP) from 0.25 to 1.0 mmol/l].

Results

We have previously shown that human fibroblasts express the P2X₇ receptor and that this receptor is coupled to plasma membrane permeability increases, Ca²⁺ influx and intracellular accumulation of microvesicles mainly originating from the Golgi apparatus ([18] and Fig. 1C, arrows). Long incubation in high glucose (22 mmol/l) strongly potentiated P2X₇ receptor-dependent microvesiculation and in addition triggered a considerable sprouting of neurite-like cellular processes rich in varicosities. Brief (1-h) stimulation by ATP or by the P2X₇ agonist BzATP triggered an expansion of the cytoplasmic vesicle compartment that occurred at a lower stimulant dose in fibroblasts kept at higher (Fig. 1F, arrows) compared with lower glucose (Fig. 1B) concentration. In the presence of 1 mmol/l BzATP high glucose concentration induced shrinkage and condensation in about 50 % of the cells (Fig. 1G, arrows) and eventual detachment from the substrate. A prolonged (24-h) stimulation with BzATP caused the appearance of several neurite-like podosomes protruding from the surviving cells (Fig. 1H, arrows). Microvesicle formation and process elongation were fully reversible upon removal of the purinergic agonist or by treatment with the P2X₇ antagonist KN62 (not shown).

Stimulation of the P2X₇ receptors is known to trigger apoptosis of several cell types [23–25], human fibroblasts are, however, an exception as we were unable to detect any cytotoxic effect dependent on P2X₇ activation in fibroblasts grown in 5 mmol/l glucose, a similar result to another study [18]. On the contrary, and in keeping with the potentiation of other P2X₇-dependent responses, high glucose concentration sensitized fibroblasts to the pro-apoptotic effect of ATP or its analogue BzATP. Stimulation with the maximum (5 mmol/l) ATP dose caused a considerable clumping and condensation of nuclear chromatin (Fig. 2D, arrows) that was eventually released into the extracellular milieu. A similar pro-apoptotic activity, albeit at lower concentrations, was also exerted by BzATP (not shown). Note that even the mere incubation in high glucose concentration, with no ATP added, caused some chromatin clumping (Fig. 2C). To further support the pro-apoptotic activity of P2X₇ stimulation in high glucose concentration, we also measured ATP-dependent activation of caspase 3. This cysteine protease was activated to a higher

level in fibroblasts kept in 22 than in 5.5 mmol/l glucose (Fig. 3). Caspase activation was strongly reduced by the specific P2X₇ inhibitor, oxidized ATP (not shown).

We then investigated the effect of high glucose concentrations on the release of the pro-inflammatory cytokine IL-6. In our previous work we showed that extracellular ATP caused IL-6 release from fibroblasts primed with phorbol myristate acetate (PMA) and bacterial endotoxin (LPS) [18]. High glucose concentration enhanced the release of this cytokine both under basal and stimulated conditions (Fig. 4). We also found ATP to be weakly but clearly effective to stimulate IL-6 secretion at the lowest doses used (10 and 30 µmol/l) and this potentiating effect was reduced at higher concentration, a finding suggesting involvement, at least in part, of P2Y receptors.

To test whether an enhanced activity of the P2X₇ receptor could be responsible for these changes, we investigated ATP-dependent plasma membrane depolarization and intracellular Ca²⁺ increases, two responses known to be dependent on P2X₇ stimulation. The ATP median effective concentration (EC₅₀) was not appreciably changed by high glucose as it was 800 and 880 µmol/l for fibroblasts kept in 22 or 5.5 mmol/l glucose, respectively, in the presence or absence of extracellular Ca²⁺ (Fig. 5). On the contrary, the maximum response was enhanced when assessed in the presence or absence of extracellular Ca²⁺. High glucose concentration sensitized fibroblasts to the depolarizing effect of the selective P2X₇ agonist BzATP (Fig. 5), especially at the lower agonist doses. Both curves obtained at 5.5 and 22 mmol/l glucose reached saturation at about the same BzATP concentration (0.5–1.0 mmol/l). The $[Ca^{2+}]_i$ increase in response to ATP was also enhanced by high glucose concentration (Fig. 6); basal $[Ca^{2+}]_i$ on the contrary was not different in fibroblasts grown in normal or high glucose concentrations [resting levels of 77 ± 15 ($n = 21$) and 73 ± 11 ($n = 21$) in normal or high glucose concentrations, respectively]. To check if P2Y responses were potentiated in high glucose concentrations, we used as a stimulant the nucleotide CTP that previous studies [18] had shown to be the best agonist for Ca²⁺ release from stores in these fibroblast populations. It has been shown previously

Fig. 1A–H. Morphological alterations caused by high glucose concentration. Control fibroblasts grown at 5.5 mmol/l glucose (A), 5.5 mmol/l glucose plus 0.5 mmol/l BzATP for 2 h (B), 5.5 mmol/l glucose plus 1 mmol/l BzATP for 2 h (C), 5.5 mmol/l glucose plus 1 mmol/l BzATP for 24 h (D), control fibroblasts grown at 22 mmol/l glucose (E), 22 mmol/l glucose plus 0.5 mmol/l BzATP for 2 h (F), 22 mmol/l glucose plus 1 mmol/l BzATP for 2 h (G), 22 mmol/l glucose plus 1 mmol/l BzATP for 24 h (H). Magnification, 250 ×

Fig. 2A–D. Effect of high glucose concentration on nuclear fragmentation. Fibroblasts grown at 5.5 mmol/l glucose (**A**), 5.5 mmol/l glucose plus 5 mmol/l ATP for 6 h (**B**), 22 mmol/l glucose (**C**), 22 mmol/l glucose plus 5 mmol/l ATP for 6 h (**D**). At the end of this incubation cells were stained with 100 μ mol/l ethidium bromide. Arrows point to nuclear clumping and fragmentation. Magnification, 360 $\times$

that CTP is equipotent with the other nucleotides at the *Xenopus* P2Y receptor [26], although it is an unusual stimulus for mammalian P2Y receptors.

In search for a mechanism responsible for this enhanced sensitivity, we investigated P2X₇ receptor expression in fibroblasts kept under either experimental conditions. Receptor expression, measured at the mRNA and protein level was, however, not altered by the standard number (six) of in vitro passages in high glucose concentrations (Fig. 7). If anything, a prolonged (30 in vitro passages) incubation in high glucose concentration caused a reduction in P2X₇ expression (Fig. 7). This serendipitous observation offered a useful control (P2X₇-less fibroblasts) to check that morphological changes triggered by BzATP were not due to chelation of extracellular divalent cations because down-modulation of P2X₇ was paralleled by an increased refractoriness to microvesicle formation and to apoptosis.

We then analysed the cellular distribution of the P2X₇ receptor by immunofluorescence with a specific antibody. In fibroblasts kept in high glucose concentrations, a polyclonal anti-P2X₇ Ab decorated ring-like membrane structures mainly localized at the periphery of the cell (Fig. 8C). Such P2X₇ receptor aggregates were only seldom observed at physiological glucose concentrations (Fig. 8).

Discussion

It was thought until recently that the P2X₇ purinergic subtype was only expressed by immune cells. It is, however, clear that many more cells and tissues express P2X₇, albeit to a lower level than immune cells [27]. Human fibroblasts express P2X₇ at the mRNA and protein level and are responsive to P2X₇ selective ligands such as BzATP. Although in immune cells P2X₇ has been associated with specific functions, in fibroblasts the key question is still that of the physiological significance of such an ion channel. Until not long ago it was doubted that ATP was secreted into the extracellular milieu in amounts sufficient to activate the P2X₇ receptor but now it is becoming increasingly evident that release of ATP by non-lytic mechanisms is the rule rather than the exception [4, 28–31], and this nucleotide is obviously released ex-

Fig. 3. Effect of physiological and high glucose concentration on caspase 3 activation. Cells were stimulated with 5 mmol/l ATP for increasing intervals, lysed with 0.1 % Triton X-100 and incubated for the indicated time in the presence of the caspase substrate. Data are averages $\pm$ SD of triplicate measurements from a single experiment repeated on three different occasions. Size of symbols exceeded that of the error bars. $\bullet$ 5.5 mmol/l, $\blacksquare$ 5.5 mmol/l + ATP, $\circ$ 22 mmol/l, $\square$ 22 mmol/l + ATP

Fig. 4. Effect of hyperglycaemia on IL-6 release. Cells were pretreated with PMA (100 nmol/l) and LPS (1 μ g/ml) for 2 h and then challenged with increasing ATP concentrations for 1 h. At the end of this incubation supernatants were withdrawn and assayed for IL-6 content. Alternatively, samples were treated with PMA or LPS alone or received no stimulant (C, control). Open bars, 5.5 mmol/l glucose. Closed bars, 22 mmol/l glucose. Data are averages $\pm$ SD of six measurements from three separate experiments

tracellularly at sites of tissue damage, for example during the inflammatory reaction. If we take into consideration that activity of plasma membrane ecto-ATPases is reported to be down-modulated at inflammatory sites [32], then it is likely that extracellular ATP can accumulate.

A

B

C

Fig. 5A–C. ATP dose-dependency curves of plasma membrane depolarization. Fibroblasts were incubated in the fluorometer cuvette at 37 °C at a concentration of 5×10^5 cells/ml in the presence of 100 nmol/l bis-oxonol and stimulated with increasing ATP (A, B) or BzATP (C). In A and C the incubation medium contained 1 mmol/l Ca^{2+} . In B, the incubation medium contained no added Ca^{2+} and 0.5 mmol/l EGTA. Depolarization was expressed as percentage of the maximum triggered by 30 mmol/l KCl. Data are averages $\pm$ SD of triplicate measurements from a single experiment repeated on three different occasions. $\blacktriangle$ 5.5 mmol/l glucose, $\blacksquare$ 22 mmol/l glucose

Although it was known since 1977 that mouse-transformed fibroblasts expressed a permeabilizing ATP receptor [33], now known to be P2X₇, very little information was available on human fibroblast primary cultures. Fibroblasts are well known for being

Fig. 6A, B Effect of high glucose concentration on $[Ca^{2+}]_i$ increases mediated by ATP (**A**) and CTP (**B**). Fibroblasts were incubated in the fluorometer cuvette at 37°C at a concentration of 10^6 cells/ml and stimulated with increasing nucleotide doses. Stimulated $[Ca^{2+}]_i$ concentration was measured at peak increase. Data are averages $\pm$ SD of triplicate determinations from a single experiment repeated on three different occasions $\blacktriangle$ 5.5 mmol/l glucose, $\blacksquare$ 22 mmol/l glucose

the major producers of extracellular matrix but also a cell type actively involved in the synthesis of inflammatory mediators and in tissue repair [34]. Furthermore, they share several characteristics with vascular smooth muscle cells, participating in the regulation of vascular tone [35]. They also participate in the development of the early steps of atherosclerotic damage and have an important role in the pathogenesis of the typical changes in extracellular matrix [36] observed in diabetes. For these reasons, fibroblasts have also been proposed as a useful *ex vivo* model for the investigation of cellular responses in diabetic subjects [37].

We and others have previously shown that high glucose concentration potentiates several cell responses such as plasma membrane receptors-depen-

Fig. 7A, B P2X₇ expression at 5.5 and 22 mmol/l glucose. RT-PCR (**A**) of P2X₇ mRNA and immunoblot (**B**) of P2X₇ protein extracted from human macrophages (lanes 1), fibroblasts grown in 5.5 mmol/l glucose for 6 (lanes 2) and 30 (lanes 4) passages or fibroblasts grown in 22 mmol/l glucose for 6 (lanes 3) and 30 (lanes 5) passages. Note that the relevant band is the upper one; the lower is an unidentified cross-reacting band

dent increases in $[Ca^{2+}]_i$ or secretion of proinflammatory cytokines such as IL-6 and TNF [38, 39]. The P2X₇ receptor has a large Ca^{2+} -increasing activity and is also coupled to release of IL-6 from human fibroblasts. Thus we wondered whether responses to ATP could also be affected by hyperglycaemia. Our results show relation between hyperglycaemia and P2X₇ receptor localization and function. On the one hand, incubation in high glucose concentration did not appreciably change the level of expression of this receptor but on the other it greatly altered its cellular localization causing the formation of receptor aggregates localized at the periphery of the cell or at sites of cell contact. Mechanism of formation and function of such aggregates are not known but we would not be surprised if they were responsible for the enhanced sensitivity to ATP of fibroblasts exposed to high glucose concentration.

Fig. 8 A–C. P2X₇ receptor distribution at 5.5 and 22 mmol/l glucose. Fibroblasts incubated only with the secondary Ab. (A) incubated in 5.5 (B) or 22 mmol/l (C) glucose. Samples were processed and stained as described in Materials and methods. Arrows indicate ring-like structures selectively stained by the anti-P2X₇ Ab. Magnification, 900 ×

Our observations could also offer a contribution to the understanding of the pathogenesis of atherosclerotic plaque. Current interest in atherosclerosis research is largely focused on the relation between lipid accumulation and inflammatory cell recruitment and subsequent cellular communication by cytokines and growth factors in the evolving atherosclerotic plaque [40]. In the plaque, a cytokine and growth factor network operates and regulates lipid metabolism,

vascular contraction, smooth muscle cell migration and proliferation.

Sensitization to ATP could have a relevant role in the mechanism by which hyperglycaemia causes microvascular damage in diabetic patients, as on the one hand at low nucleotide concentration it increases secretion of IL-6, thus generating a proinflammatory microenvironment in the vessel wall and on the other at high nucleotide concentration it triggers apoptosis, thus exacerbating tissue damage. This suggests a causal role for these receptors in plaque formation, providing a new pathogenetic mechanism to explain the accelerated and more pronounced atherosclerotic damage in diabetic patients [41, 42].

In this view, the development of more selective receptor blockers will help to better understand the physiological role of this receptor in many inflammatory and chronic degenerative diseases including Type II diabetes.

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