Rapid Publication

Glucose Induces β-Cell Apoptosis Via Upregulation of the Fas Receptor in Human Islets

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In autoimmune type 1 diabetes, Fas–to–Fas-ligand (FasL) interaction may represent one of the essential pro-apoptotic pathways leading to a loss of pancreatic β-cells. In the advanced stages of type 2 diabetes, a decline in β-cell mass is also observed, but its mechanism is not known. Human islets normally express FasL but not the Fas receptor. We observed upregulation of Fas in β-cells of type 2 diabetic patients relative to nondiabetic control subjects. In vitro exposure of islets from nondiabetic organ donors to high glucose levels induced Fas expression, caspase-8 and -3 activation, and β-cell apoptosis. The effect of glucose was blocked by an antagonistic anti-Fas antibody, indicating that glucose-induced apoptosis is due to interaction between the constitutively expressed FasL and the upregulated Fas. These results support a new role for glucose in regulating Fas expression in human β-cells. Upregulation of the Fas receptor by elevated glucose levels may contribute to β-cell destruction by the constitutively expressed FasL independent of an autoimmune reaction, thus providing a link between type 1 and type 2 diabetes. Diabetes 50:1683–1690, 2001

Type 2 diabetes manifests itself in individuals who lose the ability to produce sufficient quantities of insulin to maintain normoglycemia in the face of insulin resistance (1). Indeed, the contribution of a relative insulin deficiency to the establishment of overt diabetes is now widely accepted (2–5). The ability to secrete adequate amounts of insulin depends on β-cell function and mass. The endocrine pancreas has a remarkable capacity to adapt to conditions of increased insulin demand (e.g., in pregnancy, obesity, and cortisol or growth hormone excess) by increasing its functional β-cell mass; only 20% of the individuals under these conditions fail to adapt and become diabetic with time (5). Long-term adaptation of the β-cell mass to conditions of increased demand occurs by increasing the β-cell number through hyperplasia and neogenesis (5,6). However, β-cell expansion can be offset by concomitant apoptosis (7,8).

In a previous study, we analyzed β-cell turnover in pancreases of Psammomys obesus, a rodent with a natural tendency toward diet-induced type 2–like diabetes, initially characterized by hyperinsulinemia, but progressing to hypoinsulinemia and reduced β-cell mass (8). Analysis of β-cell turnover in P. obesus during nutrition-induced transition from normoglycemia to hyperglycemia revealed an initial and transient increase in β-cell replication that was followed by a prolonged increase in the number of apoptotic β-cells, leading to a marked reduction in the functional β-cell mass. Elevated glucose concentrations directly induced β-cell apoptosis in cultured islets from diabetes-prone P. obesus, but not in islets from normal rats (8,9). Glucose-induced β-cell proliferation was observed in both rat (10) and P. obesus islets; however, the latter showed only a limited capacity. It is not known whether elevated glucose concentrations can also adversely affect β-cell turnover in human islets and, if it can, by which mechanism.

In type 1 diabetes, the failure of the islet is already detectable at the onset of hyperglycemia because of β-cell apoptosis (11–13). However, the precise mechanisms leading to β-cell destruction remain unclear. The cell death receptor Fas (CD95) is able to signal apoptosis via an intracellular death domain (14). Cytokines can induce upregulation of Fas expression on β-cells, making them susceptible to apoptosis in the presence of agonistic anti-Fas antibodies, or interaction with Fas-ligand (FasL)–expressing T-cells (15,16) as well as neighboring β-cells (17). Nonobese diabetic (NOD) mice develop spontaneous autoimmune diabetes, but Fas-deficient NOD mice (NODIrpr) are protected against the disease (18,19). Therefore, Fas has been postulated to play an important role in the β-cell demise of type 1 diabetes. However, the role of Fas in diabetes has been challenged by several studies (20,21). Islet grafts from Fas-deficient NOD mice are protected only marginally when grafted into diabetic mice (20). Furthermore, Thomas et al. (21) detected only few, if any, Fas-expressing β-cells in islets of NOD mice close to the onset of hyperglycemia.

We hypothesized that Fas expression and activation may constitute an immune-independent event induced by transient hyperglycemic excursions. If true, this process may not be limited to type 1 diabetes but may also be
present in type 2 diabetes. Indeed, using cultured islets derived from normal individuals, we show here that pathologically elevated glucose concentrations induce apoptosis in human β-cells and that this is initiated by upregulation of the Fas receptor.

RESEARCH DESIGN AND METHODS

Islet isolation and culture. Islets were isolated from the pancreases of eight organ donors at the Division of Endocrinology and Diabetes, University Hospital of Zurich, as previously described (22). The donors, aged 17, 26, 38, 50, 56, 60, 61, and 67 years, were heart-beating cadaver organ donors, and none had a previous history of diabetes or metabolic disorders. For long-term in vitro studies, the islets were cultured on extracellular matrix-coated plates derived from bovine corneal endothelial cells (Novamed, Jerusalem), allowing the cell to attach to the dishes and spread, preserving their functional integrity (23–24). Islets were cultured in CMRL 1066 medium containing 100 units/ml penicillin, 100 µg/ml streptomycin, and 10% fetal calf serum (Gibco, Gaithersburg, MD). Two days after plating, when most islets were attached and had begun to flatten, the culture medium was changed to CMRL containing 5.5, 11.1, or 33.3 mmol/l glucose. In some experiments, islets were cultured with 2 µg/µl interleukin-1β (R&D Systems, Minneapolis, MN) or 500 ng/ml anti-Fas antibody (ZB4; MBL, Nogoya, Japan).

β-cell replication. For β-cell proliferation studies, a monoclonal antibody against the human Ki-67 antigen was used (Zymed, San Francisco, CA). Ki-67 is a nuclear antigen expressed by proliferating cells and is used as a marker for late G1, S, G2, and M phases of the cell cycle (9,25). After washing with phosphate-buffered saline (PBS), cultured islets were fixed in 4% paraformaldehyde (30 min, room temperature) followed by permeabilization with 0.5% Triton X-100 (4 min, room temperature). Afterward, islets were incubated for 1 h at room temperature with monoclonal mouse anti–Ki-67 antibody diluted 1:10, followed by detection using a streptavidin-biotin-peroxidase complex (Histostain-Plus Kit; Zymed). Subsequently, islets were incubated for 30 min at 37°C with guinea pig anti-insulin antibody diluted 1:50 (Dako, Carpinteria, CA), followed by a 10-min incubation with a 1:10 dilution of fluorescein conjugated rabbit anti–guinea pig antibody (Dako).

β-cell apoptosis. The free 3-OH strand breaks resulting from DNA degradation were detected by the terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) technique (26). Islet cultures were fixed and permeabilized as described above, then they underwent the TUNEL assay, which was performed according to the manufacturer's instructions (in situ cell death detection kit AP; Boehringer Mannheim, Mannheim, Germany). The preparations were then rinsed with Tris-buffered saline and incubated (10 min, room temperature) with 5-bromo-4-chloro-indolyl phosphate/nitro blue tetrazolium liquid substrate system (Sigma). Thereafter, islets were incubated with a guinea pig anti-insulin antibody as above, followed by detection using the streptavidin-biotin-peroxidase complex (Zymed) (Histostain-Plus Kit; Zymed) or by incubation (2 h, 37°C) with a rabbit anti–cleaved-caspase-3 antibody (1:50 dilution, D 175, Cell Signaling, Beverly, MA), followed by incubation with horseradish-peroxidase–linked anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Afterward, islets were washed in PBS, followed by a 10-min incubation with fluorescein isothiocyanate-conjugated donkey anti–rabbit antibody (1 µg/ml; Dako) and observed through a microscope.

Detection of Fas- and FasL-expressing β-cells. Pancreases from routine necropsies were fixed in formalin, followed by paraffin embedding. Sections were deparaffinized and dehydrated, and then they were endogenous peroxidase–blocked by submersion in 0.3% H2O2 for 15 min. For FasL detection, the sections were incubated in methanol for 2 min. Islet cultures were fixed and permeabilized as described above. Tissue sections and cultured islets were double-labeled for Fas receptor or for FasL and insulin by 1-h exposure to 10 µg/ml anti-Fas antibody (1:25 dilution, NOK-1; Transduction Laboratories, Lexington, KY) or by incubation (2 h, 37°C) with anti-Fas antibody (1:50 dilution; Transduction Laboratories, Lexington, KY) followed by incubation (2 h, 37°C) with anti–caspase-3 antibody (1:100 dilution; ImmunoResearch Laboratories, West Grove, PA). For analysis of FasL, Fas, caspase-3, and caspase-8, islets were suspended in 10 ml Tris-HCl, pH 7.4, 0.3% bovine serum albumin, followed by detection using the streptavidin-biotin-peroxidase complex (Zymed) or donkey anti–mouse antibody (1:50 dilution; Jackson ImmunoResearch). Mitochondria were labeled with DAPI (diluted 1:1000) and visualized using a confocal laser scanning microscope.

RESULTS

Modulation of human β-cell proliferation and apoptosis by elevated glucose concentrations. Human islets, isolated from pancreases of heart-beating donors and cultured on extracellular matrix-coated plates, were exposed to elevated glucose concentrations for 5 days. Analysis of β-cell nuclei for DNA fragmentation (TUNEL-
positive) (Fig. 1A-1 and A-2) revealed a 2.4- and 3.5-fold increase in islets cultured at medium glucose concentrations of 11.1 and 33.3 mmol/l, respectively, relative to islets at 5.5 mmol/l glucose (Fig. 1B-1). A typical feature of the DNA fragmentation in β-cells was the occasional appearance of TUNEL-positive nuclei in doublets, suggestive of postmitotic apoptosis. The TUNEL reaction may also stain necrotic cells; therefore, in parallel to the TUNEL reaction, we used the DNA-binding dye propidium iodide to assess the effect of glucose on necrosis. Exposure of islet cultures to increasing glucose concentrations (from 5.5 to 33.3 mmol/l) did not lead to propidium iodide uptake into the cultured cells (not shown). Moreover, triple-immunostaining for DNA fragmentation, insulin, and cleaved caspase-3 demonstrated cleaved caspase-3 in the TUNEL-positive β-cells (Fig. 1A-2 insert), confirming an apoptotic process. To exclude a nonspecific effect of the high concentration of d-glucose (33.3 mmol/l), osmolarity was corrected with the metabolically inactive L-glucose. A 5-day exposure to 27.8 mmol/l L-glucose together with 5.5 mmol/l d-glucose resulted in a β-cell DNA fragmentation rate similar to that induced by 5.5 mmol/l d-glucose alone. The time-course of the effect of 33.3 mmol/l glucose on DNA fragmentation reveals a significant increase in β-cell death after 1 day of exposure to the high glucose level and an increased number of TUNEL-positive β-cells persisting throughout the 10 days of the study (2.0-, 7.4-, 3.0-, 4.0-, and 5.0-fold increase after 1, 3, 5, 7, and 10 days of treatment, respectively, compared with 5.5 mmol/l glucose) (Fig. 1B-2).

Exposure of cultured human islets to elevated glucose concentrations for 5 days decreased the number of proliferating (Ki-67-positive) β-cells (Fig. 1A-3 to A-6). Proliferation was reduced by 42 and 61% in medium containing 11.1 and 33.3 mmol/l glucose, respectively, relative to islets at 5.5 mmol/l glucose (Fig. 1B-3). Exposure of the islets to 27.8 mmol/l L-glucose together with 5.5 mmol/l d-glucose did not change the baseline proliferative activity observed with 5.5 mmol/l d-glucose alone. An initial increase of 1.5- and 2.5-fold inKi-67-positive β-cells was observed after a 1- and 3-day exposure, respectively, to 33.3 mmol/l glucose (relative to islets at 5.5 mmol/l glucose); longer exposure times resulted in a marked inhibition of the β-cells’ proliferative capacity (three- and twofold decrease after 5 and 10 days, respectively, of exposure to 33.3 mmol/l glucose) (Fig. 1B-4).

These studies indicate that, unlike the long-term increase in β-cell death generated in human islets by a continuous exposure to elevated glucose levels, β-cell proliferative capacity exhibited only a transient increase followed by a prolonged decrease.

Glucose induces Fas expression and activation in human β-cells. We next studied the cellular mechanism of glucose-induced apoptosis in human β-cells; specifically, we investigated the possible involvement of the Fas receptor pathway. Exposure of cultured human islets to increasing glucose concentrations (from 5.5 to 33.3 mmol/l glucose) for 5 days resulted in a dose-dependent increase in Fas receptor expression in the β-cells, as determined by double-immunostaining (with anti-Fas and anti-insulin antibodies) islets plated on extracellular matrix-coated dishes (Fig. 2A). The large majority of the Fas-positive cells observed were insulin-positive. However, a few cells were Fas-positive and insulin-negative. These cells can be insulin-depleted β-cells or non-β-cells. The effect of elevated glucose on Fas expression was verified by Western blot analysis (Fig. 2B). Glucose-dependent cleavage of procaspase-3 to activated caspase-3 was also identified by Western blotting (Fig. 2B), supporting the idea that glucose-induced DNA fragmentation, as determined by the TUNEL assay, represents apoptotic β-cell death. Treatment of islets with 33.3 mmol/l glucose induced activation of caspase-8 (Fig. 2C). Because caspase-8 is the most upstream caspase in the Fas apoptotic pathway, its activation by glucose further supports a role for Fas in glucose-induced apoptosis. Changes in glucose concentration in the culture medium did not induce release of cytochrome c from the mitochondria to the cytosol (Fig. 2D), suggesting that glucose-dependent apoptosis is independent of the mitochondrial→cytochrome c pathway in human islets.

Constitutive expression of FasL in human islets and upregulation of the Fas receptor in islets of hyperglycemic type 2 diabetic patients. Loweth et al. (17) showed constitutive expression of FasL in human islets. However, this finding was not confirmed by two subsequent studies (30,31). The negative results were imputed to the specificity of the antibody used, which in subsequent studies has been reported to produce a false-positive reaction (32). We tested an antibody shown to be specific for FasL (31,32) and found substantial amounts of FasL in the human β-cells of control and diabetic patients (Fig. 3, panels 1–6). The expression of FasL was verified by immunostaining of isolated islets (not shown) as well as by Western blot analysis (Fig. 2B). Moreover, the presence of FasL mRNA transcripts was verified by in situ hybridization in the β-cells of diabetic (Fig. 3, panels 7 and 8) and normal individuals (Fig. 3, panels 9 and 10), whereas the exocrine pancreas was negative. For controls, we used a digoxigenin-labeled sense probe and found no signal (Fig. 3, panel 11). We concluded that the previously reported failure to detect FasL was probably caused by differences in the preparation of the pancreas sections because it was possible to detect FasL in the β-cell only after performing an antigen retrieval procedure with methanol, as described by Loweth et al. (17).

The Fas receptor is not expressed in normal human pancreatic islets (17,31). However, based on our in vitro studies, we anticipated its expression in the islets of type 2 diabetic patients as a result of repeated hyperglycemic episodes. Expression of Fas was therefore studied in sections of pancreases from five poorly controlled type 2 diabetic patients, all with documented fasting blood glucose >8 mmol/l. Double-immunostaining and mRNA in situ hybridization of the pancreatic sections for Fas and insulin revealed localization of the Fas receptor to the β-cells (Fig. 3, panels 13, 14, 19, and 20). Fas expression could not be detected in β-cells of nondiabetic control subjects (Fig. 3, panels 15, 16, 21, and 22).

Glucose-induced β-cell apoptosis and impaired proliferation are mediated by Fas-FasL interaction in human islets. To examine whether the induction of apoptosis and impaired proliferation by glucose is caused by interaction between constitutively expressed FasL and...
FIG. 1. Characterization of the effect of elevated glucose concentrations on human β-cell apoptosis and proliferation. A: Islets were exposed for 5 days to media containing 5.5 mM D-glucose (panels 1, 3, and 5) or 33.3 mM D-glucose (panels 2, 4, and 6). Triple-immunostaining (panels 1 and 2) for insulin (orange), DNA fragmentation by the TUNEL assay (black), and cleaved caspase-3 (green, bottom insert). Detection of β-cell proliferation with anti–Ki-67 (red; panels 3 and 4) and with anti-insulin antibody (green; panels 5 and 6). The white arrows mark nuclei stained positive for the TUNEL reaction, and the black arrows mark nuclei stained positive for Ki-67. Original magnification ×400. B: Relative number of TUNEL-positive (panel 1) and Ki-67–positive β-cells (panel 3) per islet after 5-day culture in 5.5, 11.1, and 33.3 mM D-glucose or in 5.5 mM D-glucose plus 27.8 mM L-glucose, normalized to control incubations at 5.5 mM D-glucose alone (100%; in absolute value: 0.53 TUNEL-positive β-cells per islet and 1.16 Ki-67–positive β-cells per islet). TUNEL-positive (panel 2) and Ki-67–positive (panel 4) β-cells per islet during 10-day culture at 5.5 mM D-glucose or 33.3 mM D-glucose. The mean number of islets scored was 141 for each treatment condition. Islets were isolated from eight heart-beating cadaver organ donors. Results are shown as means ± SE. *P < 0.01 relative to islets at 5.5 mM D-glucose; **P < 0.01 relative to islets at 33.3 mM D-glucose.
FIG. 2. Glucose induces Fas expression and caspase activation in human β-cells. A: Double-immunostaining for the Fas receptor (panels 1, 3, and 5) and insulin (panels 2, 4, and 6) in human islets cultured on extracellular matrix–coated dishes and exposed for 5 days to media containing 5.5 mmol/l glucose (panels 1 and 2), 11.1 mmol/l glucose (panels 3 and 4), or 33.3 mmol/l glucose (panels 5 and 6). Original magnification ×400, with higher magnification of β-cells stained for the Fas receptor (panel 5a) and insulin (panel 6a). Immunostaining for the Fas receptor (panels 7 and 8) in untransfected human malignant glioma cells (negative control) (panel 7) and in cell transfected with human Fas cDNA (positive control) (panel 8). B: Immunoblotting of Fas receptor, FasL, procaspase-3, and activated caspase-3. Human islets cultured in suspension at 5.5, 11.1, or 33.3 mmol/l glucose were analyzed after a 36-h incubation. Human fibroblast derived from foreskin was used as a positive control for Fas. The antibodies were blotted on the same membrane after stripping. C: Caspase-8 activation. D: Subcellular localization of cytochrome c in human islets cultured at 5.5 or 33.3 mmol/l glucose for 36 h or in the presence of 0.5 mmol/l Palmitic acid (positive control). Immunoblotting of cytochrome c was performed on mitochondrial and cytosolic fractions. Each experiment was repeated three times.
upregulated Fas, we used the antagonistic anti-Fas antibody ZB4. Because interleukin-1β–induced β-cell apoptosis has recently been shown to involve an association between Fas and its ligand (17,31), ZB4 was also tested for its effect on interleukin-1β–mediated injury. In human islets, interleukin-1β increased β-cell apoptosis and de-

FIG. 3. Constitutive expression of FasL in human islets and upregulation of the Fas receptor in islets of hyperglycemic type 2 diabetic patients. Double-immunostaining for FasL (orange; panels 1, 3, and 5) and insulin (green; panels 2, 4, and 6) in tissue sections of pancreas from a patient with type 2 diabetes—without (panels 1 and 2) and with a preabsorption by FasL peptide (negative control; panels 5 and 6)—and from a nondiabetic patient (panels 3 and 4). mRNA in situ hybridization for FasL (red; panels 7, 9, and 11) double-immunostained for insulin (green; panels 8, 10, and 12) in a tissue section of a pancreas from a patient with type 2 diabetes—using anti-sense probe (panel 7) and sense probe (negative control; panel 11)—and from a nondiabetic patient using anti-sense probe (panel 9). Double-immunostaining for the Fas receptor (orange; panels 13, 15, and 17) and insulin (green; panels 14, 16, and 18) in tissue sections of a pancreas from a patient with type 2 diabetes—without (panels 13 and 14) and with a preabsorption by Fas peptide (negative control; panels 17 and 18)—and from a nondiabetic patient (panels 15 and 16). mRNA in situ hybridization for Fas (red; panels 19, 21, and 23) double-immunostaining for insulin (green; panels 20, 22, and 24) in a tissue section of a pancreas of a patient with type 2 diabetes—using anti-sense probe (panel 19) and sense probe (negative control; panel 23)—and of a nondiabetic patient using anti-sense probe (panel 21). Original magnification ×400.
pressed FasL on neighboring receptors, which can interact with the constitutively ex-

In the present study, we show that increased glucose concentration by itself induces apoptosis in human pancreatic β-cells. The mechanism underlying glucose-induced β-cell death involves the upregulation of Fas receptors, which can interact with the constitutively expressed FasL on neighboring β-cells. Fas-FasL interaction leads to cleavage of procaspase-8 to caspase-8. Activated caspase-8, the most upstream caspase in the Fas apoptotic pathway, promotes caspase-3 activation and DNA fragmentation (33). We demonstrate here that caspase-8 and -3 are indeed activated by high glucose in human islets; the mitochondrial apoptotic pathway does not appear to be involved.

In contrast to that seen in human islets, an increase in glucose concentration to 11 mmol/l in rat islets promotes β-cell survival (8,9,29,34). When glucose concentrations were further increased, glucose proved to be pro- or anti-apoptotic, depending on culture conditions. The difference in glucose sensitivity between human and rat islets can be explained by the mechanism of glucose-induced β-cell apoptosis. Human islets constitutively express FasL (17) (Fig. 3), whereas islets from 2- to 3-month-old rats—the age at which rats are usually investigated—do not express FasL (35). Thus, whereas glucose-induced Fas in human β-cells interact with FasL expressed in adjacent β-cells, Fas ligation does not occur in young rats. These interspecies differences raise the possibility that differences in sensitivity to glucose may also exist among individuals of the same species. Indeed, although glucose was capable of inducing β-cell apoptosis in each sample from the eight organ donors studied, large variations were observed in the response. It may be postulated that both genetic background and age may determine the susceptibility to glucose-induced β-cell apoptosis: type 2 diabetes occurs more often in older people with an inherited predisposition.

Elevated glucose concentrations induced Fas expression in almost all β-cells; however, apoptosis was observed in only a few cells. An interesting feature of the glucose-induced apoptosis was the appearance of fragmented nuclei doublets, suggestive of postmitotic apoptosis. Therefore, susceptibility to apoptosis via Fas activation may be increased in proliferating cells. Because glucose also induces β-cell proliferation, a relationship between induction of proliferation and apoptosis seems plausible. In line with this suggestion, exposure of β-cells to elevated glucose concentrations induced a short-lasting increase in proliferation accompanied by long-lasting β-cell apoptosis. The same sequence is also observed in vivo during the evolution of diabetes (1,8,36,37). Thus, reduced compensatory proliferative response coupled with increased β-cell death in response to hyperglycemia may well contribute to the progressive decline of β-cell mass in diabetic patients.

So far, induction of Fas receptors on β-cells was considered to be limited to type 1 diabetes in response to cytokines during the process of autoimmune destruction. Here, we demonstrate that glucose may directly induce the Fas receptor on human β-cells, leading to apoptosis due to interaction with the constitutively expressed FasL of surrounding β-cells. Moreover, we observed the expression of Fas in islets of type 2 diabetic patients. Therefore, a similar mechanism for β-cell destruction probably exists in both type 1 and 2 diabetes. However, in type 1 diabetes, islet cell destruction may not result solely from activation of the Fas pathway but also from the action of cytokines and cytolytic perforin/granzyme released from cytotoxic T-cells. Nevertheless, our results underscore the importance of tight glucose control in limiting β-cell destruction in all diabetic patients as well as in patients undergoing islet transplantation.

**FIG. 4.** Effect of blockade of the Fas receptor on glucose- and interleukin-1β-induced β-cell DNA fragmentation and proliferative activity. Human islets were cultured on extracellular matrix-coated dishes for 5 days in 5.5 or 33.3 mmol/l glucose alone (control) or in the presence of interleukin-1β (IL-1), antagonistic Fas antibody (ZB4), or both. Results are means ± SE of the relative number of TUNEL-positive (panel 1) and Ki-67–positive β-cells (panel 2) per islet normalized to control incubations at 5.5 mmol/l glucose alone (100%; in absolute values: 0.18 TUNEL-positive β-cells per islet and 1.16 Ki-67–positive β-cells per islet). The mean number of islets scored was 33 for each treatment condition. *P < 0.001 relative to islets at 5.5 mmol/l glucose; **P < 0.05 relative to control at the same glucose concentration; †P < 0.05 relative to interleukin-1β–treated cells.
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