Bimodal Effect on Pancreatic β-Cells of Secretory Products From Normal or Insulin-Resistant Human Skeletal Muscle

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OBJECTIVE—Type 2 diabetes is characterized by insulin resistance with a relative deficiency in insulin secretion. This study explored the potential communication between insulin-resistant human skeletal muscle and primary (human and rat) β-cells.

RESEARCH DESIGN AND METHODS—Human skeletal muscle cells were cultured for up to 24 h with tumor necrosis factor (TNF)-α to induce insulin resistance, and mRNA expression for cytokines was analyzed and compared with controls (without TNF-α). Conditioned media were collected and candidate cytokines were measured by antibody array. Human and rat primary β-cells were used to explore the impact of exposure to conditioned media for 24 h on apoptosis, proliferation, short-term insulin secretion, and key signaling protein phosphorylation and expression.

RESULTS—Human myotubes express and release a different panel of myokines depending on their insulin sensitivity, with each panel exerting differential effects on β-cells. Conditioned medium from control myotubes increased proliferation and glucose-stimulated insulin secretion (GSIS) from primary β-cells, whereas conditioned medium from TNF-α–treated insulin-resistant myotubes (TMs) exerted detrimental effects that were either independent (increased apoptosis and decreased proliferation) or dependent on the presence of TNF-α in TM (blunted GSIS). Knockdown of β-cell mitogen-activated protein 4 kinase 4 prevented these effects. Glucagon-like peptide 1 protected β-cells against decreased proliferation and apoptosis evoked by TMs, while interleukin-1 receptor antagonist only prevented the latter.

CONCLUSIONS—Taken together, these data suggest a possible new route of communication between skeletal muscle and β-cells that is modulated by insulin resistance and could contribute to normal β-cell functional mass in healthy subjects, as well as the decrease seen in type 2 diabetes. *Diabetes* **60:1111–1121, 2011**

Regulation of β-cell function and mass represent a critical issue for understanding diabetes, a disease characterized in its two major forms by a relative or absolute deficiency in the number of pancreatic β-cells and their function. Type 1 (insulin-dependent) diabetes thus results from autoimmune destruction of pancreatic β-cells (1), and it is now recognized that there is decreased β-cell mass and function in type 2 diabetes (2) but the precise underlying mechanism remains to be determined. Insulin resistance is obviously a key player in type 2 diabetes, but it also plays a larger role in the disease process of type 1 diabetes than is commonly recognized (3).

Far from being an inert tissue in terms of interorgan communication, it is now recognized that skeletal muscle can secrete so-called myokines, which can impact both favorably and unfavorably on the function of distant organs/tissues. Interleukin (IL)-6 is the prototype myokine and was identified as the first long-searched-for muscle contraction–induced factor (4). It has been suggested that IL-6 impacts on β-cell function, but more detailed studies are needed to clarify the direct impact of this cytokine on pancreatic β-cells in health and disease, especially because the α-cell has been identified as a major IL-6 target in islets (5). Adiponectin, first identified as a secretory product of adipocytes, has also been shown to be secreted by skeletal muscles (6) and can impact positively on pancreatic β-cell function and survival (7).

More generally, studies performed in vitro have confirmed that various cytokines and chemokines can impact positively or negatively on the function, survival, and proliferation of β-cells (8), including IL-1β (9), IL-6 (5,10), adiponectin (7), and tumor necrosis factor-α (TNF-α), (11) as well as chemokine (C-C motif) ligand 5 (CCL5), monocyte chemoattractant protein-1 (MCP-1), IL-8, and CXC chemokine ligand (CXCL10) (12,13). This raises the possibility of beneficial or detrimental effects of cytokines on β-cells depending on concentration and biological context.

Skeletal muscle plays a major role in whole-body substrate homeostasis in the postprandial state, and impaired action on this tissue contributes to the pathological condition of insulin resistance (14). The recent identification of skeletal muscle as an endocrine organ that produces and releases myokines expands our knowledge of how the nervous, endocrine, and immune systems contribute to the maintenance of homeostasis, also when challenged by changing physiological demands (4). Given that skeletal muscle is the prevalent organ in the human body in terms of its mass, we decided to explore if skeletal muscle with

distinct insulin sensitivity can differentially impact on β-cell function.

During the last years, multiple studies have been performed to understand the mechanism of insulin resistance (15). We showed previously that TNF-α can induce insulin resistance in human skeletal muscle by altering the insulin signaling pathway (16), providing a unique system for molecular dissection in vitro (17). The physiological source of increased circulating levels of TNF-α in type 2 diabetes remains unclear, but it does not appear to be the muscle itself (18). TNF-α is, however, believed to be a major cytokine involved in the "conversation" between adipose tissue and muscle with increased levels in obesity and type 2 diabetes possibly contributing to insulin resistance in skeletal muscle (19).

With this background in mind, we have used this validated in vitro model of skeletal muscle insulin resistance to explore putative conversation between skeletal muscle and β-cells. A different panel of myokines is secreted by normally sensitive or insulin-resistant myotubes that impact in a beneficial or detrimental way, respectively, on β-cell function, proliferation, and survival.

RESEARCH DESIGN AND METHODS

Antibodies and reagents. The antibodies and reagents were as follows: human TNF (hTNF)-α (R&D Systems Europe Ltd., Abingdon, U.K.); anti-phospho–Akt-(Ser473), anti-phospho–ERK-1/2-(Thr202/Tyr204) (New England Biolabs, Beverly, MA); anti-phospho–(Ser/Thr)-AS160, anti-AS160, anti-Akt, anti-ERK, anti–insulin receptor substrate (IRS)-2 (Cell Signaling Technology, Beverly, MA); antiactin (Sigma, Buchs, Switzerland); anti–IRS-1 (gift from Dr. M.F. White; Children's Hospital, Harvard University, Boston, MA); Ethanercept (Wyeth Pharmaceuticals SA, Zoug, Switzerland); 5-bromo-2-deoxyuridine (BrdU) and transferase-mediated dUTP nick-end labeling (TUNEL) kits (Roche, Mannheim, Germany); IL-1RA (Kineret), Amgen (Breda, the Netherlands); and glucagon-like peptide 1 (GLP-1) (Bachem, Bubendorf, Switzerland).

Human skeletal muscle cells. *Rectus abdominus* muscle was obtained with the informed consent of donors during scheduled abdominal surgery (Geneva Hospital and Danish Muscle Center approved the study protocols NAC 06–093 and jr. nr. H-A-2007–0016). This particular slow-twitch fiber type of skeletal muscle was chosen for ease of surgical access as well as insulin-sensitive glucose uptake using in vitro–derived myotubes. The satellite cells were isolated from the muscle biopsies by trypsin digestion and grown in Ham's F10 medium with 20% FCS and antibiotics. Confluent myoblasts were allowed to fuse and differentiate into myotubes in Dulbecco's modified Eagle's medium, 2% FCS, and antibiotics. Human myotubes were used 7 days after induction of differentiation (when most cells are multinucleated) and expressed specific human skeletal muscle markers (17).

Oligo GEArray experiments. Total RNA was extracted with the QIAshredder and RNeasy Mini Kit (QIAGEN, Basel, Switzerland). The UTP-biotinylated cRNA probe was synthesized with the TrueLabeling-AMP 2.0 Kit (SuperArray, Frederick, MD) and purified with the ArrayGrade cRNA Cleanup Kit (SuperArray). The membranes (focused on human inflammatory cytokines and receptors [OHS-011 from SABiosciences, Frederick, MD]) were hybridized following the manufacturer's instructions.

Human chemokine and cytokine antibody arrays. Human chemokine + human cytokine V arrays (Ray Biotech, Norcross, GA) were used following the manufacturer's instructions, and images were quantified using Multi Gauge software with intensity normalized to internal controls.

Rat primary β-cell purification. Animals were treated according to protocols approved by the State Commissioner on Animal Care. Islets of Langerhans were isolated by collagenase digestion of pancreas from male Wistar rats (150–200 g) followed by Ficoll purification (20). β-Cells sorted by fluorescence-activated cell sorter were plated on 804G extracellular matrix (20,21).

Human primary β-cell purification. Human islets were provided by the Islet Cell Resource Centers of Milan (Italy), Geneva (Switzerland), and Lille (France). Human β-cells were sorted by fluorescence-activated cell sorter to >90% purity following labeling with Newport Green (21).

Insulin secretion assay and insulin content. Cells were incubated for 1 h at 37°C with Krebs-Ringer bicarbonate buffer with Hepes (KRBH) containing 2.8 mmol/L glucose (basal secretion), followed by 1 h at 37°C with KRBH containing 16.7 mmol/L glucose (stimulated secretion) as previously described (22). Insulin in incubation buffers and cell extracts was measured by radioimmunoassay and secretion expressed as percentage total insulin content.

Detection of apoptosis and proliferation. Cell death was measured by TUNEL assay. Proliferation was assessed by BrdU incorporation (22).

RNA interference mediated silencing of MAP4K4. Knockdown of MAP4K4 in rat primary β-cells was achieved by transfection with small interfering RNA as previously described (11). All other standard methods were as described previously (22).

Presentation of data and statistics. Data are mean ± SEM for 3–5 independent experiments (2 replicates for each independent experiment for insulin secretion, BrdU incorporation, and TUNEL assay). Statistical significance for differences was evaluated by one- or two-way ANOVA, as appropriate, using Fisher least significant differences test for post hoc determination.

RESULTS

Impact of TNF-α treatment on human skeletal muscle cells. Human myotubes were cultured for 24 h with 20 ng/mL TNF-α. Cells treated with TNF-α showed decreased IRS-1 protein expression (Fig. 1A), and a diminution of both IRS-1 tyrosine (85% vs. untreated control, $P < 0.01$) and Akt (Ser497) phosphorylation (54% vs. untreated control, $P < 0.01$) after insulin stimulation (representative Western blot) (Fig. 1B). This validates the experimental model, confirming that TNF-α induced insulin resistance as expected (17). The impact of TNF-α on myotube death was also examined. At a concentration of 20 ng/mL, TNF-α (as used in the current study) had no impact on human skeletal muscle cell death at any time up to 72 h (Fig. 1C). However, there was a significant decrease of cell death at 24 h using 10 ng/mL TNF-α, but an increase at 40 ng/mL (Fig. 1D), albeit with <2% of the cells being TUNEL-positive even under these latter conditions.

Myokine expression and secretion from human skeletal muscle cells (myotubes) treated with or without TNF-α. Human myotubes were cultured for up to 24 h with (TNF-α–treated insulin-resistant myotubes [TMs]) or without (control myotubes [CMs]) 20 ng/mL TNF-α. After 8 h, mRNA expression of 128 candidate genes for inflammatory cytokines and their receptors were analyzed using Oligo nucleotide array membranes. Insulin-resistant myotubes (+ TNF-α) showed increased expression of 19 cytokine genes (Fig. 2A). This was confirmed and further quantified by quantitative RT-PCR (Fig. 2B). To determine whether such cytokines were released to the medium, conditioned media were collected after 24 h, and candidate cytokines were measured. Using an antibody array allowing for detection of 79 proteins in this large family, we were able to confirm that CCL5, CXCL10, CXCL2, IL-6, IL-8, CCL2, CCL7, CXCL6, CXCL3, and CXCL1 were detectable and increased in the medium from TNF-α–treated human myotubes when compared with untreated myotubes (Fig. 3A and B), with increases ranging from 2.7-fold (osteoprotegrin) to 87-fold (Gro-α). Absolute concentrations of IL-8, IL-6, and TNF-α in TMs were measured independently ($n = 2$): IL-8, 3.50 ng/mL; IL-6, 2.5 ng/mL; TNF-α, 18 ng/mL. TNF-α was measured to confirm its stability in the conditioned medium (initial concentration 20 ng/mL).

Impact of conditioned medium from human myotubes with different insulin sensitivity on primary sorted β-cells. We had shown that TNF-α itself inhibits glucose-stimulated insulin secretion (GSIS) but does not impact growth or survival of β-cells (11). We now wished to determine the effect on these β-cell parameters of conditioned medium from TMs or CMs. To this end, β-cells were treated for 48 h with either CMs or TMs, with BrdU added during the last 24 h. Only rat β-cells were used to measure proliferation, because adult human β-cells fail to proliferate to any meaningful extent in vitro (21). Because we
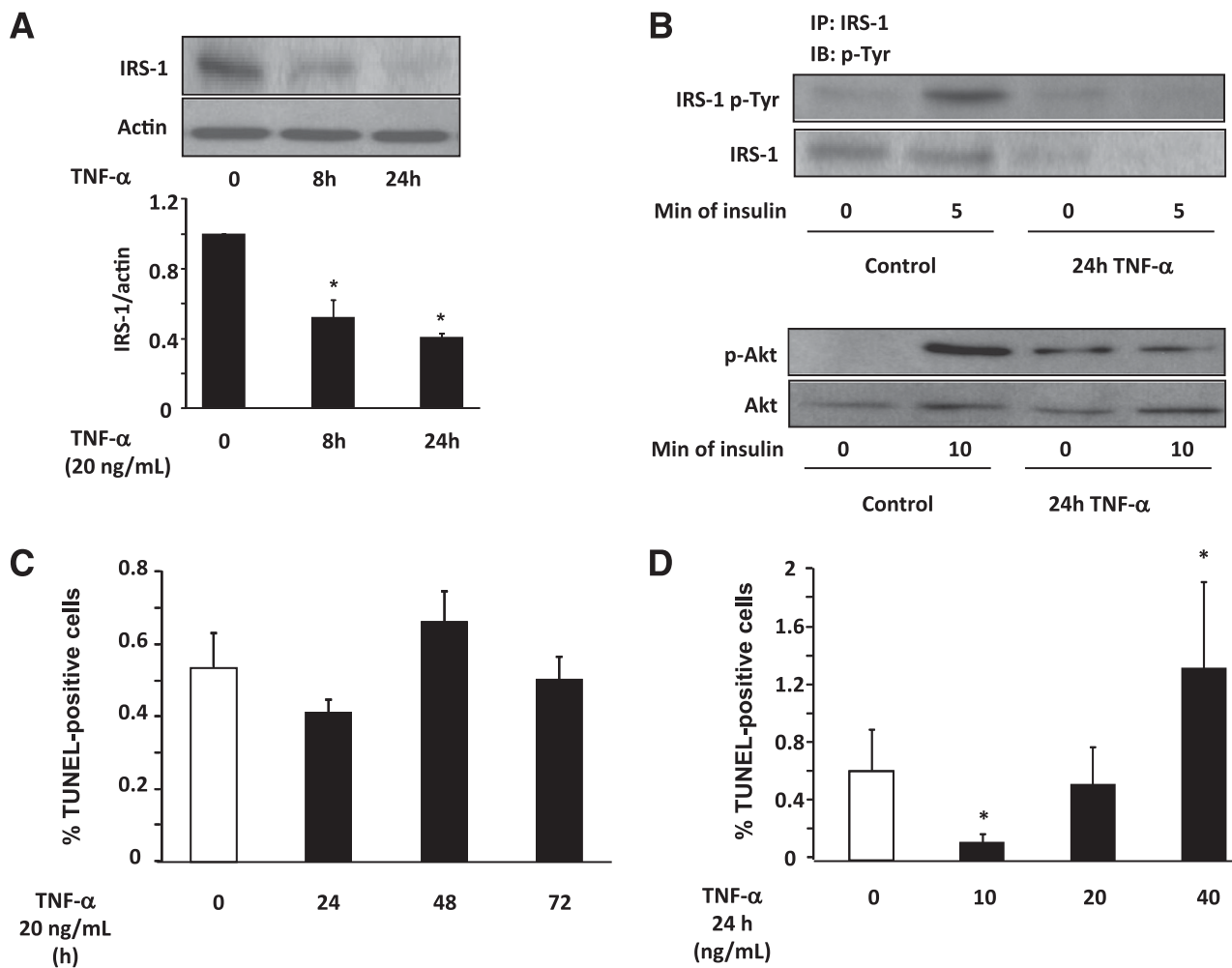
FIG. 1. Effect of TNF-α on insulin sensitivity and apoptosis in human skeletal muscle cells. *A*: Human myotubes were cultured 24 h ± 20 ng/mL TNF-α, and IRS-1 protein expression was measured by Western blot (normalized to actin). Mean ± SE, 5 independent experiments. *B*: 24 h after incubation with TNF-α, human skeletal muscle cells were treated with 100 nmol/L insulin either for 5 min to induce IRS-1 tyrosine phosphorylation or 10 min to measure Akt phosphorylation. Representative immunoblots are shown for IRS-1 tyrosine (Tyr) phosphorylation (*upper panel*) and Akt Ser479 phosphorylation (*lower panel*). *C*: Effect of different times of TNF-α treatment (20 ng/mL) on human skeletal muscle cell death measured by TUNEL assay. $n = 5$ independent experiments. *D*: Effect of different TNF-α concentrations (24 h) on human primary skeletal muscle cell death measured by TUNEL assay. $n = 5$ independent experiments; *$P < 0.05$ vs. control. IP, immunoprecipitation; IB, immunoblotting.

documented the continued presence of TNF-α in TMs (see above), an additional control condition involved CMs "spiked" with 20 ng/mL TNF-α immediately before adding it to the β-cells (CM + TNF-α). There was a dramatic decrease in rat primary β-cell proliferation treated with TMs when compared with CMs or CM + TNF-α (Fig. 4*A*). This is the first direct demonstration that products secreted from insulin-resistant (but not control) skeletal muscle can impact negatively on β-cells. Interestingly, CMs induced a significant increase in β-cell proliferation when compared with the control condition without CMs, suggesting a beneficial, mitogenic effect of products released by untreated (normally insulin-sensitive) myotubes (Fig. 4*A*).

Both rat and human primary (sorted) β-cells were used to evaluate the impact of TMs (24 h) on cell death and insulin secretion. Neither CMs nor the addition of TNF-α to such medium (CM + TNF-α) had any effect on β-cell apoptosis. However, TMs induced a sixfold increase in primary β-cell death (Fig. 4*B* and *C*). GSIS was significantly decreased in both rat and human primary β-cells treated

with either TMs or CM + TNF-α with no change in basal insulin secretion (Fig. 4*D* and *E*, respectively). Using both rat and human primary sorted β-cells, CMs alone (but not CM + TNF-α) induced a significant increase in GSIS (Fig. 4*D* and *E*), confirming the beneficial effects of CMs from myotubes that were seen for proliferation of rat β-cells.

Taken together, these results document the existence of products secreted from myotubes that impact either positively or negatively on pancreatic β-cell function, proliferation, and survival.

Dual effects of conditioned medium from skeletal muscle on Akt and extracellular signal–related kinase phosphorylation in β-cells. We and others have demonstrated that activation of the insulin signaling pathway (via IRS-2) is necessary for β-cell survival, proliferation, and GSIS (22–25). In order to explore the possible impact of conditioned medium from skeletal muscle on this pathway, rat primary β-cells were cultured in the presence of CMs or TMs for 24 h. After this, cells were handled exactly as for the measurement of GSIS. Phosphorylation of both Akt and extracellular signal–related kinase (ERK)
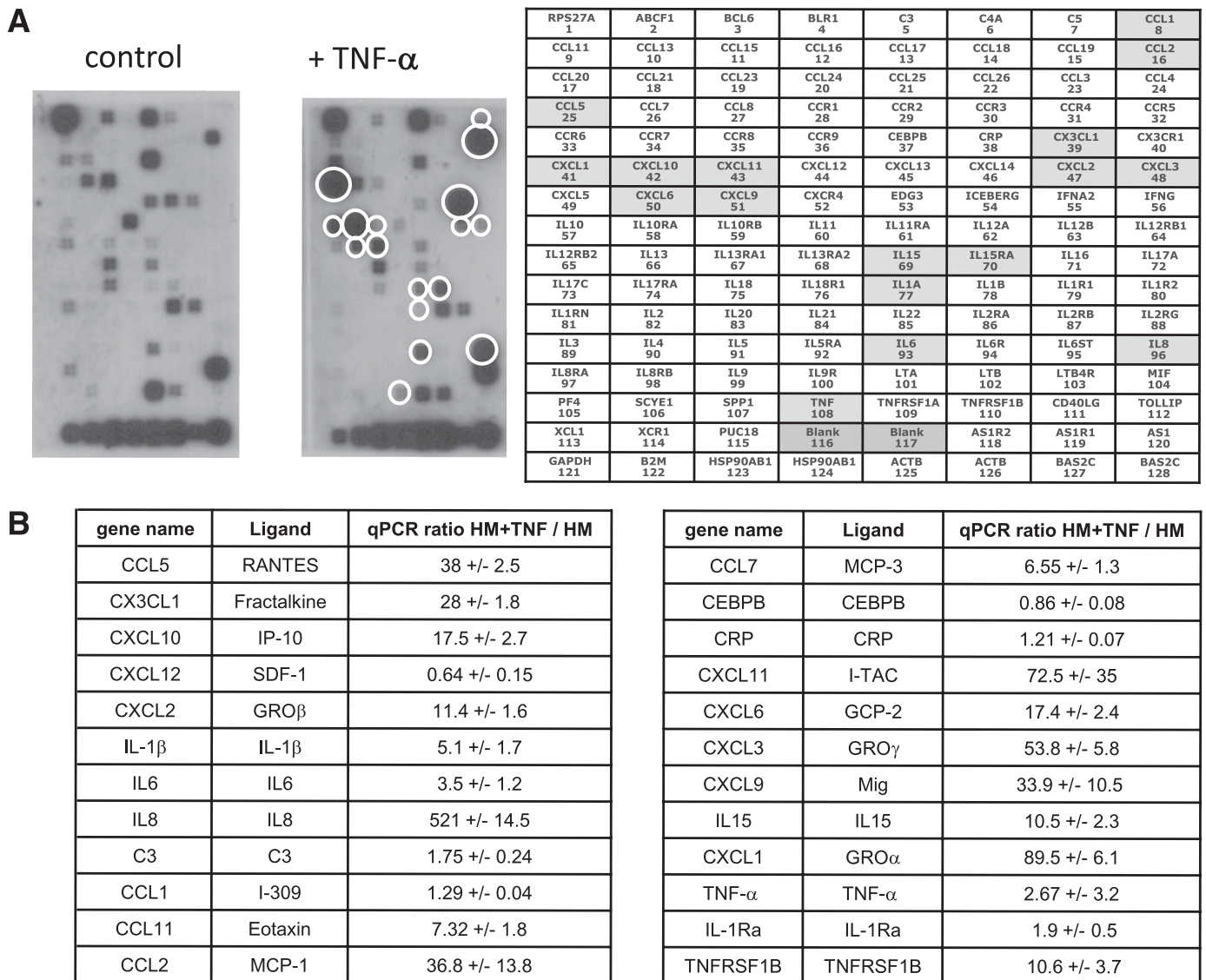
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control   + TNF-α

RPS27A 1	ABCF1 2	BCL6 3	BLR1 4	C3 5	C4A 6	C5 7	CCL1 8
CCL11 9	CCL13 10	CCL15 11	CCL16 12	CCL17 13	CCL18 14	CCL19 15	CCL2 16
CCL20 17	CCL21 18	CCL23 19	CCL24 20	CCL25 21	CCL26 22	CCL3 23	CCL4 24
CCL5 25	CCL7 26	CCL8 27	CCR1 28	CCR2 29	CCR3 30	CCR4 31	CCR5 32
CCR6 33	CCR7 34	CCR8 35	CCR9 36	CEBPB 37	CRP 38	CX3CL1 39	CX3CR1 40
CXCL1 41	CXCL10 42	CXCL11 43	CXCL12 44	CXCL13 45	CXCL14 46	CXCL2 47	CXCL3 48
CXCL5 49	CXCL6 50	CXCL9 51	CXCR4 52	EDG3 53	ICEBERG 54	IFNA2 55	IFNG 56
IL10 57	IL10RA 58	IL10RB 59	IL11 60	IL11RA 61	IL12A 62	IL12B 63	IL12RB1 64
IL12RB2 65	IL13 66	IL13RA1 67	IL13RA2 68	IL15 69	IL15RA 70	IL16 71	IL17A 72
IL17C 73	IL17RA 74	IL18 75	IL18R1 76	IL1A 77	IL1B 78	IL1R1 79	IL1R2 80
IL1RN 81	IL2 82	IL20 83	IL21 84	IL22 85	IL2RA 86	IL2RB 87	IL2RG 88
IL3 89	IL4 90	IL5 91	IL5RA 92	IL6 93	IL6R 94	IL6ST 95	IL8 96
IL8RA 97	IL8RB 98	IL9 99	IL9R 100	LTA 101	LTB 102	LTB4R 103	MIF 104
PF4 105	SCYE1 106	SPP1 107	TNF 108	TNFRSF1A 109	TNFRSF1B 110	CD40LG 111	TOLLIP 112
XCL1 113	XCR1 114	PUC18 115	Blank 116	Blank 117	AS1R2 118	AS1R1 119	AS1 120
GAPDH 121	B2M 122	HSP90AB1 123	HSP90AB1 124	ACTB 125	ACTB 126	BAS2C 127	BAS2C 128

B

gene name	Ligand	qPCR ratio HM+TNF / HM
CCL5	RANTES	38 +/- 2.5
CX3CL1	Fractalkine	28 +/- 1.8
CXCL10	IP-10	17.5 +/- 2.7
CXCL12	SDF-1	0.64 +/- 0.15
CXCL2	GROβ	11.4 +/- 1.6
IL-1β	IL-1β	5.1 +/- 1.7
IL6	IL6	3.5 +/- 1.2
IL8	IL8	521 +/- 14.5
C3	C3	1.75 +/- 0.24
CCL1	I-309	1.29 +/- 0.04
CCL11	Eotaxin	7.32 +/- 1.8
CCL2	MCP-1	36.8 +/- 13.8

gene name	Ligand	qPCR ratio HM+TNF / HM
CCL7	MCP-3	6.55 +/- 1.3
CEBPB	CEBPB	0.86 +/- 0.08
CRP	CRP	1.21 +/- 0.07
CXCL11	I-TAC	72.5 +/- 35
CXCL6	GCP-2	17.4 +/- 2.4
CXCL3	GROγ	53.8 +/- 5.8
CXCL9	Mig	33.9 +/- 10.5
IL15	IL15	10.5 +/- 2.3
CXCL1	GROα	89.5 +/- 6.1
TNF-α	TNF-α	2.67 +/- 3.2
IL-1Ra	IL-1Ra	1.9 +/- 0.5
TNFRSF1B	TNFRSF1B	10.6 +/- 3.7

FIG. 2. Effect of TNF-α on expression of cytokine genes in human skeletal muscle cells. *A*: Human myotubes were cultured 8 h ± 20 ng/mL TNF-α, and cytokine mRNA levels were monitored by hybridization to Oligo nucleotide array membranes. *White circles* show modified mRNA levels between the two conditions on representative membranes out of four different experiments. *B*: Cytokine mRNA levels were measured by quantitative RT-PCR (qRT-PCR). The data are the mean value of four independent experiments, and mRNA expression levels were normalized to cyclophilin A. HM + TNF, human skeletal muscle cells after 8-h culture with TNF-α; HM, human skeletal muscle cells after 8-h culture without TNF-α.

were stimulated by glucose, and TM pretreatment abolished this to the same extent as TNF-α on the background of CM (Fig. 5*A* and *B*) suggesting this to be TNF-α–dependent. Conversely, Akt (but not ERK) phosphorylation was increased in CM-treated β-cells under basal (but not glucose-stimulated) conditions (Fig. 5*A*), which could explain in part increased proliferation of β-cells cultured with such conditioned medium (Fig. 4*A*) (26).

Dual effects of conditioned medium from skeletal muscle on phosphorylation of substrates of Akt in β-cells. We have demonstrated that AS160 is phosphorylated in response to glucose in β-cells and is involved in GSIS as well as β-cell survival (22). As observed for Akt, TM and TNF-α treatment prevented AS160 phosphorylation in response to glucose (Fig. 5*C*). Because AS160 is just one of the multiple substrates that can be phosphorylated by Akt, we explored the total pattern of such substrates after glucose stimulation. As shown in Fig. 5*D*, glucose induced the phosphorylation of several Akt substrate proteins, while TM treatment prevented it for nearly all these proteins (Fig. 5*D*, black crosses). Given the similar pattern obtained using TM versus CM + TNF-α, this effect was again presumed to be driven by the TNF-α that was still present in TM. In the basal condition, phosphorylation of AS160 (Fig. 5*C*) and other yet-to-be-identified Akt substrate proteins (Fig. 5*D*, white crosses) were increased when primary rat β-cells were treated 24 h with CMs. Here again, these results can explain the positive impact of CM on survival and GSIS when primary rat β-cells were treated with CMs.

Dual effects of conditioned medium from myotubes on IRS-1 and -2 mRNA and protein expression in β-cells. IRS-1 and -2 have been shown to be central in both insulin production and secretion (27,28). Therefore, we explored the impact of conditioned media on IRS-1 and -2 expression. CM treatment induced an increase of protein and mRNA expression for IRS-1 and -2 (Figs. 5*E* and *F* and
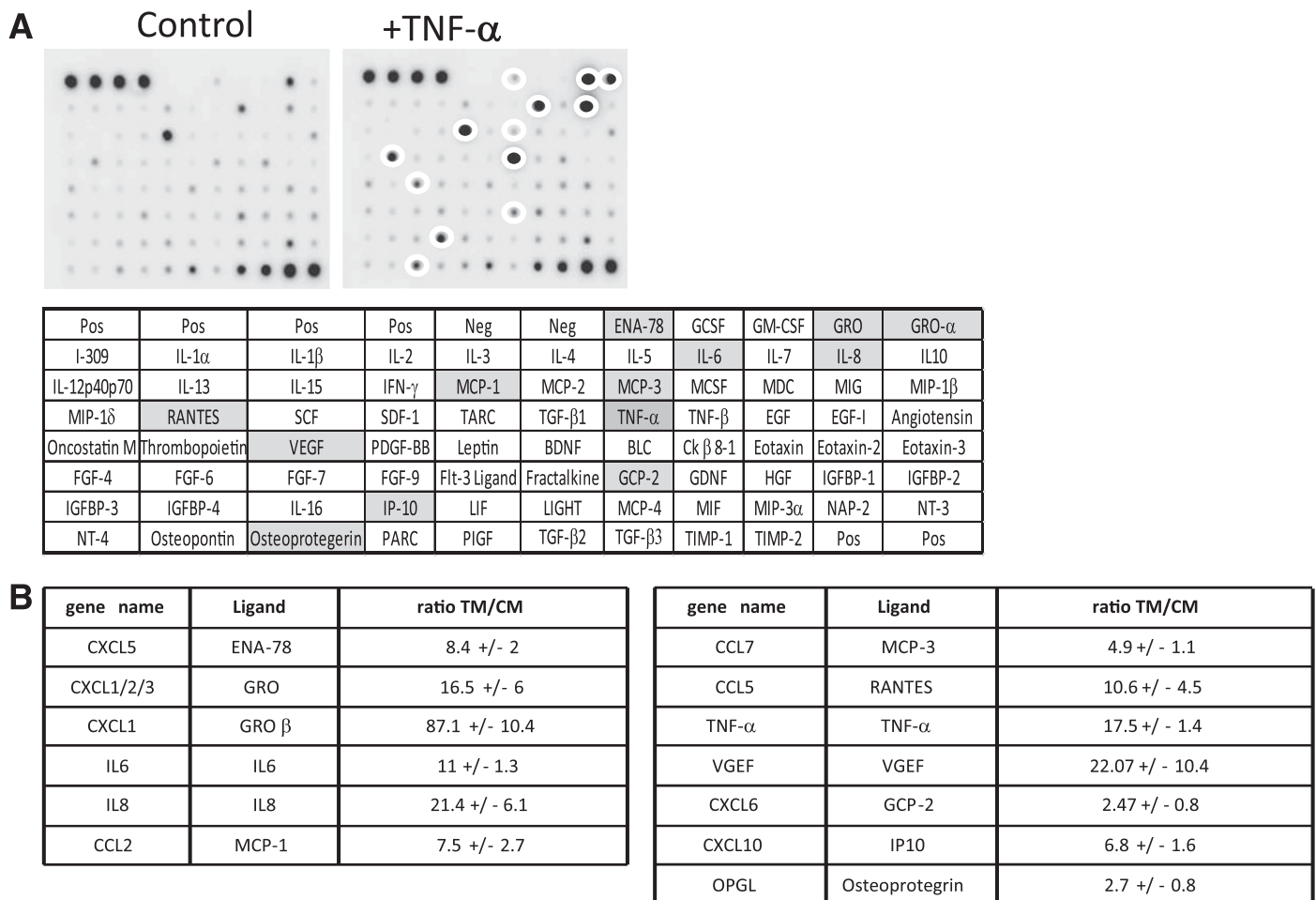
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Pos	Pos	Pos	Pos	Neg	Neg	ENA-78	GCSF	GM-CSF	GRO	GRO-α
I-309	IL-1α	IL-1β	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7	IL-8	IL10
IL-12p40p70	IL-13	IL-15	IFN-γ	MCP-1	MCP-2	MCP-3	MCSF	MDC	MIG	MIP-1β
MIP-1δ	RANTES	SCF	SDF-1	TARC	TGF-β1	TNF-α	TNF-β	EGF	EGF-I	Angiotensin
Oncostatin M	Thrombopoietin	VEGF	PDGF-BB	Leptin	BDNF	BLC	Ck β 8-1	Eotaxin	Eotaxin-2	Eotaxin-3
FGF-4	FGF-6	FGF-7	FGF-9	Flt-3 Ligand	Fractalkine	GCP-2	GDNF	HGF	IGFBP-1	IGFBP-2
IGFBP-3	IGFBP-4	IL-16	IP-10	LIF	LIGHT	MCP-4	MIF	MIP-3α	NAP-2	NT-3
NT-4	Osteopontin	Osteoprotegerin	PARC	PIGF	TGF-β2	TGF-β3	TIMP-1	TIMP-2	Pos	Pos

B

gene name	Ligand	ratio TM/CM
CXCL5	ENA-78	8.4 +/- 2
CXCL1/2/3	GRO	16.5 +/- 6
CXCL1	GRO β	87.1 +/- 10.4
IL6	IL6	11 +/- 1.3
IL8	IL8	21.4 +/- 6.1
CCL2	MCP-1	7.5 +/- 2.7

gene name	Ligand	ratio TM/CM
CCL7	MCP-3	4.9 +/ - 1.1
CCL5	RANTES	10.6 +/ - 4.5
TNF-α	TNF-α	17.5 +/ - 1.4
VGEF	VGEF	22.07 +/ - 10.4
CXCL6	GCP-2	2.47 +/ - 0.8
CXCL10	IP10	6.8 +/ - 1.6
OPGL	Osteoprotegrin	2.7 +/ - 0.8

FIG. 3. Effect of TNF-α on human skeletal muscle cell cytokine secretion. *A*: Human myotubes were cultured 24 h ± 20 ng/mL TNF-α, and cytokine levels in the conditioned media were monitored by hybridization to human chemokine + cytokine array membranes. *White circles* show modified protein levels between the two conditions on representative membranes from three different experiments. *B*: Each membrane was quantified using Multi Gauge software, and the intensity was normalized to internal positive controls for comparison. $n = 3$ independent experiments. TM, medium from human skeletal muscle cells cultured 24 h with TNF-α; CM, medium from human skeletal muscle cells cultured 24 h without TNF-α.

6*A* and *B*). This was prevented when TNF-α was added to CMs. Surprisingly, TM treatment increased IRS-1 protein expression despite the presence of TNF-α in this medium (Fig. 5*E*). There was, however, no such change in IRS-1 mRNA levels (Fig. 6*A*). By contrast, TM treatment decreased both IRS-2 protein and mRNA expression when compared with CMs, whereas TNF-α had only an impact on IRS-2 protein expression (Figs. 5*F* and 6*B*).

MAP4K4 mediates the effect of TMs on proliferation, apoptosis, and GSIS in rat primary β-cells. MAP4K4 has been shown to mediate TNF-α action in adipose tissue, skeletal muscle, and β-cells (11,17,29). In rat primary β-cells, the amount of MAP4K4 mRNA was increased by 60 and 40% after either TNF-α or TM treatment for 24 h (Fig. 6*C*). Unfortunately, there is no available antibody in rats to monitor MAP4K4 protein levels by Western blot, and it also is not possible to measure its activity. We therefore adopted an indirect method to evaluate the importance of MAP4K4 modulation by TMs. Transfection of primary rat β-cells with small interfering RNA decreased MAP4K4 mRNA expression by 60% in primary β-cells treated with TMs (11). Rat primary β-cells lacking MAP4K4 were partially resistant to TM action on proliferation (Fig. 6*D*) and totally protect against TM-induced apoptosis and its

impact on GSIS (Fig. 6*E* and *F*). These data suggest that MAP4K4 is an important mediator of TM action on pancreatic β-cells and that TM can impact on MAP4K4 activity independently of TNF-α.

Impact of IL-1RA, IL-6 receptor blockade, and GLP-1 on effects of TMs on primary rat β-cells. We show above that TMs decrease β-cell proliferation, survival, and GSIS (Fig. 4). We also show that human myotubes treated with TNF-α for 24 h show an increase of several cytokines including IL-1β (Figs. 2 and 3), which has been shown to be involved in type 2 diabetes (30,31). Therefore, we decided to explore whether pretreatment of β-cells with IL-1RA, the natural soluble IL-1 receptor antagonist, could prevent TM effects on β-cells. IL-1RA treatment failed to prevent TM action on either GSIS (data not shown) or proliferation (Fig. 7*A*), whereas TM action on cell death was prevented (Fig. 7*B*). IL-1RA treatment did also induce a decrease of proliferation in the control condition and CM-treated β-cells. This seemingly paradoxical observation can be explained by the fact that β-cells plated as here on extracellular matrix secrete low levels of IL-1β that impact positively on β-cell survival (32), which is in keeping with the established bimodal effects of this cytokine on β-cells (33).
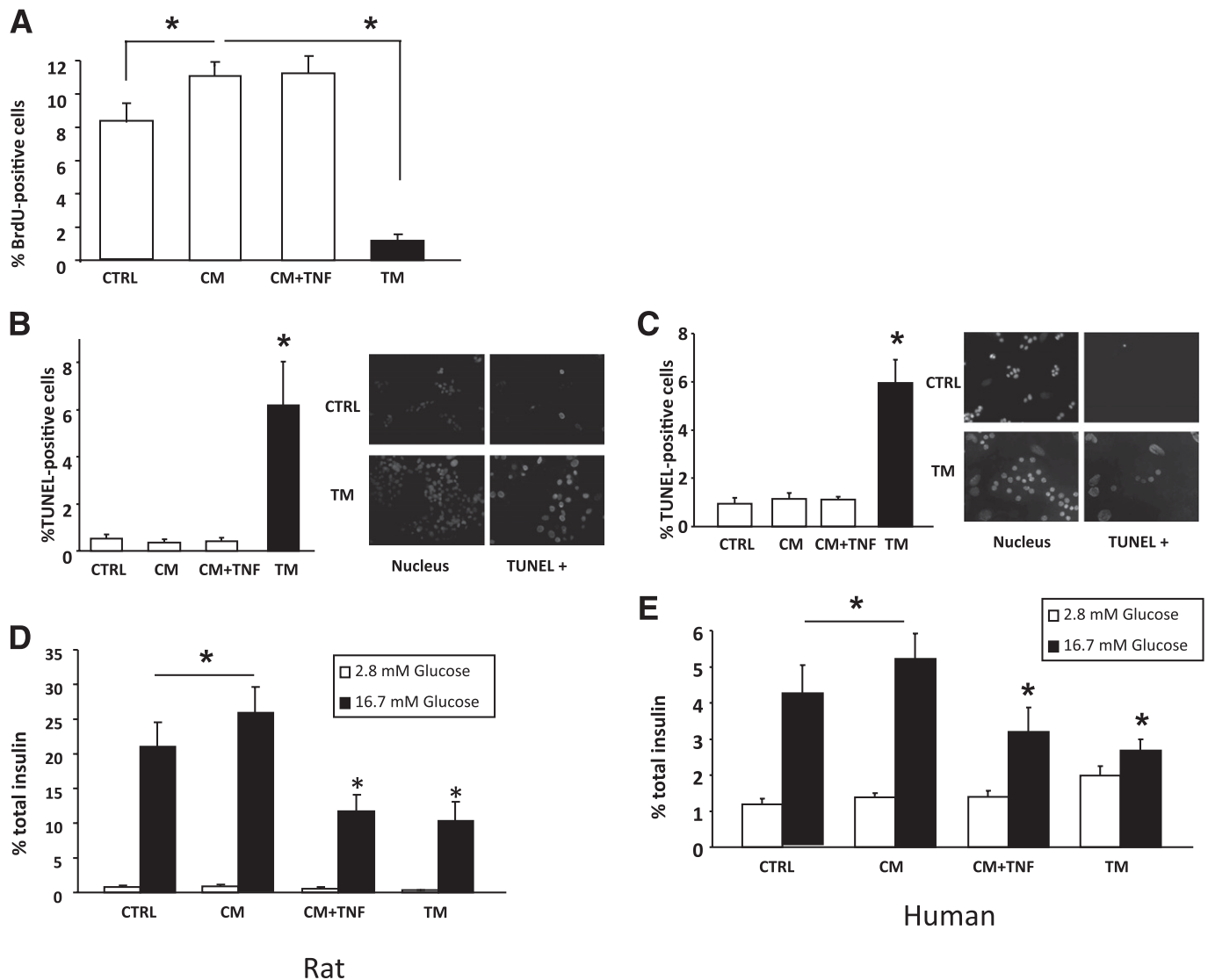
FIG. 4. Effect of conditioned medium on primary β-cell proliferation, survival, and GSIS. Conditioned medium was obtained by culturing human myotubes for 24 h with (TM) or without (CM) 20 ng/mL TNF-α. Additional control conditions were as follows: CTRL, culture medium not previously exposed to skeletal muscle cells; CM + TNF, 20 ng/mL TNF-α added to CM immediately before exposure to β-cells. *A*: Proliferation of rat primary β-cells measured by BrdU incorporation. Cells were grown under standard culture conditions (20% FCS, 11.2 mmol/L glucose) and treated for 48 h with the different conditioned media; BrdU was added for the last 24 h. β-Cells were identified by insulin immunofluorescence. *n* = 7 independent experiments. *B* and *C*: Rat and human primary β-cell apoptosis. Cell death was measured by TUNEL. *n* = 7 (rats) and *n* = 5 (human) independent experiments. *P < 0.05. *D* and *E*: Glucose-stimulated insulin secretion from rat and human primary β-cells measured during 60 min at 2.8 mmol/L glucose (*white bars* = basal secretion) following by 60 min at 16.7 mmol/L glucose (*dark bars* = stimulated secretion). Secretion is expressed as a percentage total insulin content. *n* = 7 (rats) and *n* = 5 (human) independent experiments. *P < 0.05.

It has been postulated that communication between skeletal muscle and pancreas could be mediated by IL-6 (10). To evaluate this possibility, rat primary β-cells were treated with IL-6 receptor antagonist (Sant-7) or a human anti–IL-6 receptor antibody (AF-227-NA) to block IL-6 action on β-cells. Under the present experimental conditions, neither means of IL-6 receptor blockade was able to block TM effects on rat primary β-cell proliferation, apoptosis, or GSIS (data not shown). Nevertheless, blocking the IL-6 receptor did completely inhibit the increase of β-cell proliferation evoked by CMs (data not shown).

Studies have shown that GLP-1 treatment can protect β-cells from the negative actions of cytokines (34,35) and also enhances primary rat β-cell proliferation (21). Therefore we have explored if GLP-1 treatment could protect β-cells against the detrimental effects evoked by TMs. As

expected, primary rat β-cells pretreated with GLP-1 showed an increase in proliferation under basal conditions and also after CM treatment (Fig. 7C). However, pretreatment with GLP-1 induced only a very small—albeit significant—increase in proliferation of cells treated with TMs (Fig. 7C), whereas such treatment protected totally the β-cells from TM action on apoptosis (Fig. 7D). GLP-1 treatment failed to protect against the decrease in GSIS following 24-h culture with TMs (data not shown).

TNF-α antibody blocks TM action only on GSIS. We have published that treatment with TNF-α alone can impair GSIS in primary rat and human β-cells (11) without affecting proliferation of apoptosis. Therefore, we explored directly which actions of TMs on primary rat β-cells were because of the presence of TNF-α in this conditioned medium using an antibody toward TNF-α (Ethanercept).
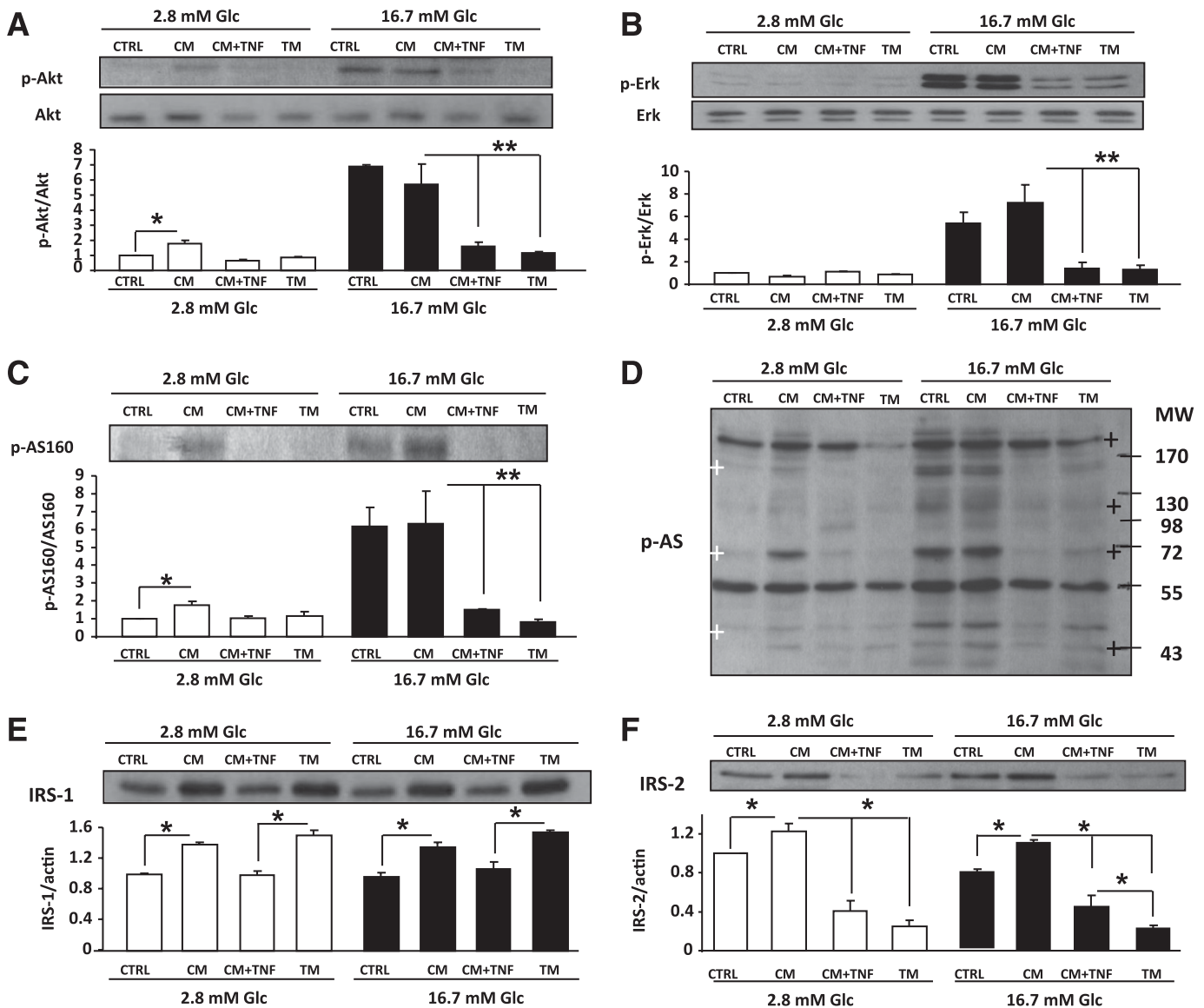
FIG. 5. Action of conditioned medium on Akt, ERK, AS160, and Akt substrates phosphorylation after glucose stimulation, or IRS-1 and -2 protein expression in rat primary β-cells. Conditioned media and abbreviations as described in the legend to Fig. 3. After 24-h culture in conditioned medium, β-cells were incubated 1 h at 2.8 mmol/L or 16.7 mmol/L glucose (Glc). *Open bars* = 2.8 mmol/L glucose; *closed bars* = 16.7 mmol/L glucose. Western blots were scanned and data normalized to total protein or actin as indicated. N = 3 independent experiments. *A*: Akt Ser 473 phosphorylation. *$P < 0.05$; **$P < 0.01$. *B*: ERK1/2 phosphorylation. **$P < 0.01$. *C*: AS160 phosphorylation. *$P < 0.05$; **$P < 0.01$. *D*: Ser/Thr phosphorylation of Akt substrates (*white crosses* = CM effect; *black crosses* = TM effect). *E* and *F*: IRS-1 (*E*) and IRS-2 (*F*) protein expression. *$P < 0.05$. CTRL, control.

Ethanercept (2 ng/mL) was able to block both TNF-α and TM action on GSIS. This was accompanied by an increase in basal secretion (Fig. 7*E*). However, trapping of TNF-α remained without effect on TM action on proliferation and apoptosis (Fig. 7*F* and *G*), confirming that these effects are because of the impact of factors secreted from TNF-α–treated myotubes and not because of TNF-α present in this conditioned medium. Interestingly, Ethanercept treatment increased apoptosis and decreased proliferation in rat β-cells cultured for 48 h with CMs (Fig. 7*F* and *G*). These results lead us to hypothesize that TNF-α at low levels in CMs could act positively by itself or in combination with other myokines on β-cell survival and proliferation. Our combined data with Ethanercept and MAP4K4 silencing reinforce the conclusion that TM action on proliferation and survival is TNF-α–independent and mediated by

MAP4K4 activation by factors secreted by insulin-resistant myotubes.

DISCUSSION

To understand the mechanism involved in type 2 diabetes development, peripheral insulin resistance and β-cell function have to be studied in parallel (36,37). Low-grade systemic inflammation is also a feature of obesity and diabetes (38), raising the hypothesis that elevated cytokine levels may contribute to insulin resistance and decreased β-cell functional mass (39). Nonmuscle-derived TNF-α contributes to insulin resistance in human skeletal muscle (16,17) and can also inhibit insulin secretion (11). Nowadays, skeletal muscle is accepted as an endocrine organ (40). This study reports that human myotubes produce and release
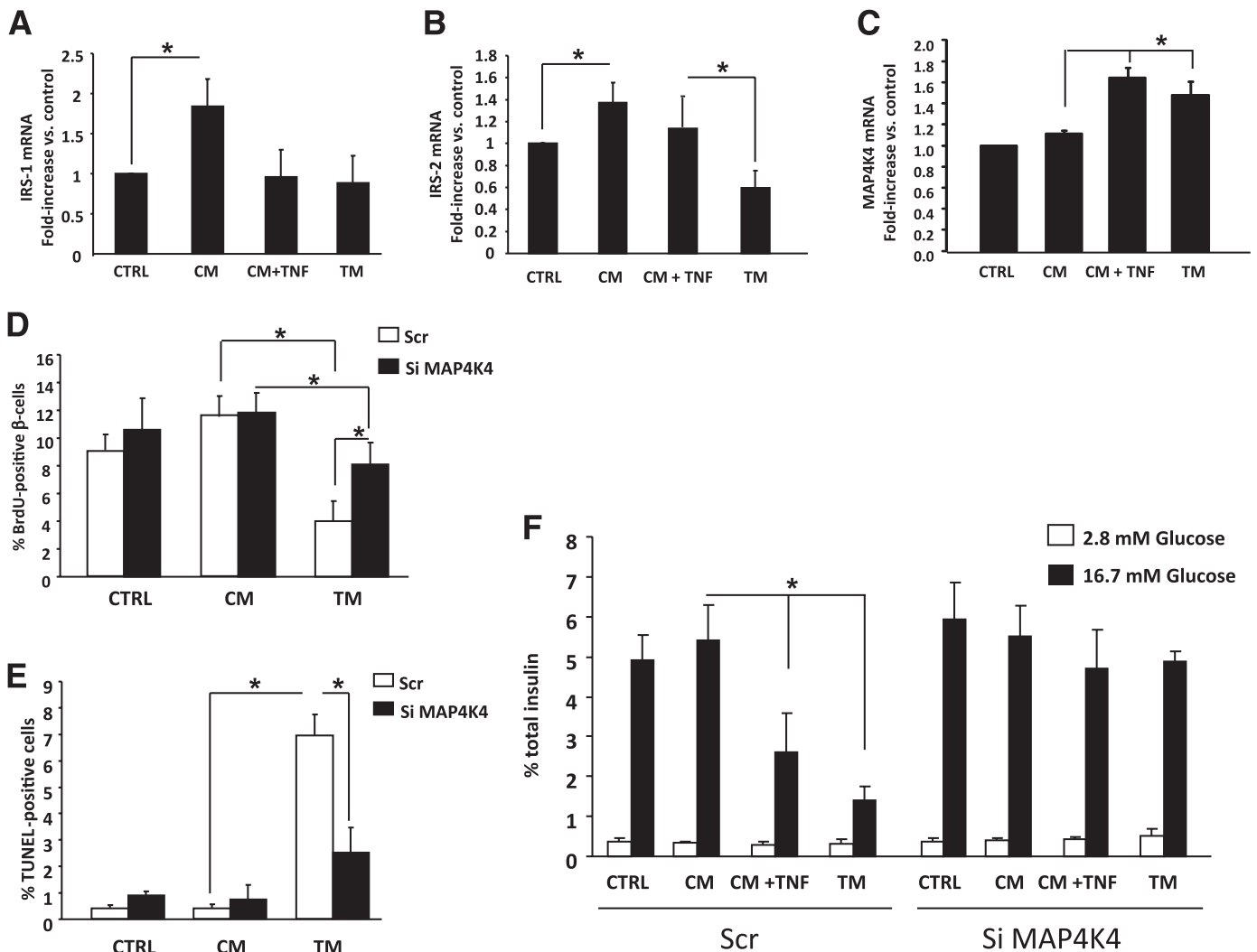
FIG. 6. Action of conditioned medium on IRS-1, IRS-2, and MAP4K4 mRNA expression and effect of MAP4K4 knockdown. Conditioned media and abbreviations as described in the legend to Fig. 3. Rat primary β-cells were used for $n = 5$ independent experiments. A–C: IRS-1, IRS-2, and MAP4K4 mRNA expression were measured by quantitative real-time RT-PCR in β-cells. *$P < 0.05$. D–F: Proliferation (BrdU incorporation) (D) and apoptosis (TUNEL) (E) were measured in β-cells transfected with scrambled (Scr) (*open bars* = Scr) or with MAP4K4 small interfering RNA (siRNA) Si MAP4K4 (*closed bars* = Si MAP4K4). F: Insulin secretion was measured using β-cells transfected with scrambled (Scr) or with Si MAP4K4 and incubated for 60 min at 2.8 mmol/L glucose (*open bars*) followed by 60 min at 16.7 mmol/L glucose (*closed bars*). *$P < 0.05$. CTRL, control.

different cytokines depending on their state of insulin sensitivity, adding to the list of previously known myokines (41,42). This was achieved using a candidate approach, and an unbiased screening would likely reveal yet more.

We have also focused on the possible endocrine impact of these myokines on sorted primary human and rat β-cells. There was bimodal action with conditioned medium from human CMs that are fully sensitive to insulin-exerted beneficial effects on β-cells with increased proliferation and GSIS, whereas that from insulin-resistant myotubes (TMs) exerted detrimental effects with increased apoptosis, and decreased proliferation and GSIS. Myokines like IL-1β, IL-6, TNF-α, CCL5, MCP-1, IL-8, and CXCL10 are good candidates for these latter, detrimental actions as they have been shown to impact negatively on β-cell function and survival (5,9–13). However, IL-RA, TNF-α trapping, and IL-6 receptor blockade failed to completely rescue β-cells from TM action, exemplifying the complex nature of the cocktail of cytokines in this conditioned

medium. Meanwhile, GLP-1 treatment was able to rescue primary β-cells from TM-mediated apoptosis, confirming its antiapoptotic properties (34). The data further indicate that lower concentrations of cytokines secreted by insulin-sensitive myotubes, as previously observed for IL-1β (33) and confirmed here for this cytokine as well as TNF-α and IL-6, could have a positive action on β-cells in keeping with bimodal action depending on concentration and the biological context (33). Nevertheless, other myokines, already identified or not, could be involved in both the positive and negative effects of CMs or TMs.

In order to gain a more detailed understanding of the underlying molecular pathways, we explored the signaling pathways impacted in β-cells by the various conditioned media and possible means to protect them from such action. In β-cells, activation of the insulin-signaling pathway is necessary to mediate glucose action (23) with IRS-2/Akt/AS160 involved in GSIS (22). Moreover, IRS-2 is known to be indispensable for β-cell function (43) while ERK activation has been shown to be involved in GSIS (44). TM
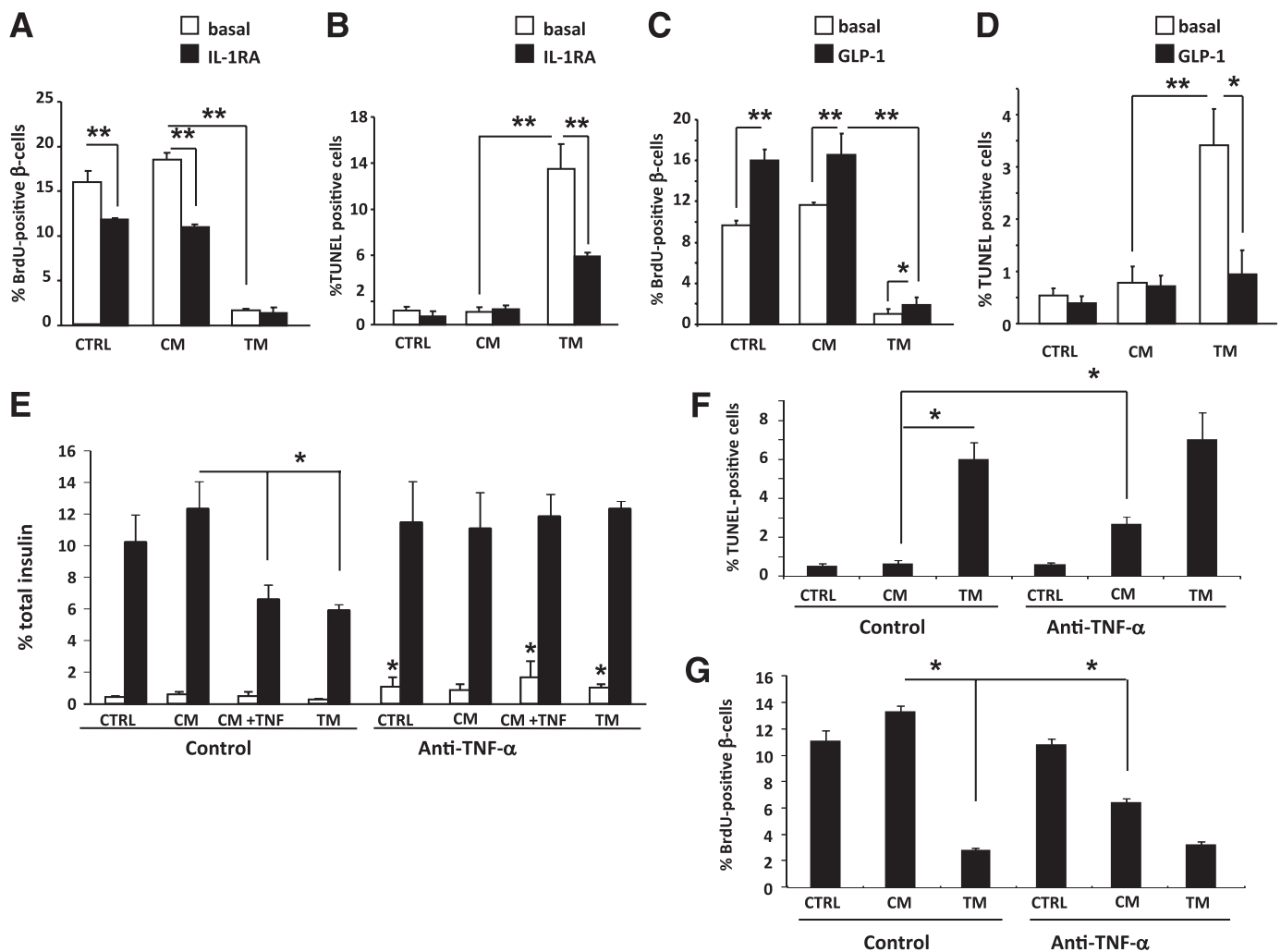
FIG. 7. Effect of IL-1RA, GLP-1, or anti–TNF-α on rat primary β-cells exposed to conditioned medium. Conditioned media and abbreviations as described in the legend to Fig. 3. Rat primary β-cells were treated 48 h with IL-1RA (1 μg/mL) and conditioned medium. *A*: BrdU was added during the last 24 h to measure proliferation. *B*: Conditioned medium added for the last 24 h and cell death was measured by TUNEL. *C*: Rat primary β-cells were treated 48 h with conditioned medium, with or without GLP-1 (100 nmol/L), and BrdU was added the last 24 h to measure proliferation. *D*: Rat primary β-cells were treated 48 h with or without GLP-1, and conditioned medium was added for the last 24 h. Cell death was measured as described. $n = 3$ independent experiments. *$P < 0.05$; **$P < 0.01$. *E*: Insulin secretion was measured using rat primary β-cells treated for 24 h with the different conditioned media with or without TNF-α blockade (2 ng/mL Ethanercept) and then incubated for 60 min at 2.8 mmol/L glucose (*open bars*) followed by 60 min at 16.7 mmol/L glucose (*closed bars*); *$P < 0.05$. *F* and *G*: Rat primary β-cells treated with the different conditioned media with or without Ethanercept and were used to assess cell death using TUNEL assay (*F*) and cell proliferation (*G*) as described. $n = 4$ independent experiments; *$P < 0.05$ for TNF-α blockade. CTRL, control.

treatment blocks glucose action on ERK, as well as Akt and its substrates including AS160. We also demonstrated that glucose-stimulated phosphorylation of a number of Akt substrates in rat primary β-cells was prevented by TMs. Only a few of these Akt substrates have been identified in muscle and adipose tissue while nothing is known in pancreatic β-cells aside from our previous work on AS160 showing that this Akt substrate was indispensable for GSIS (22). Conversely, CM treatment of primary sorted β-cells increased phosphorylation of Akt and several Akt substrates under basal conditions. This could partly explain the positive impact of CMs on primary β-cells.

Protein and mRNA levels of IRS-2 were decreased by TMs with a compensatory effect seeming to occur between IRS-1 and -2 as both TMs and CMs induced an increase of IRS-1 expression, while only CMs induced an increase of IRS-2. This supports findings in other tissues that IRS-1 and -2 have separate and nonredundant function (45).

It has been shown that insulin resistance can be rescued after MAP4K4 silencing in skeletal muscle and adipose tissue (17,46) while another study reported that silencing of MAP4K4 in macrophages can suppress systemic inflammation and therefore prevent diabetes (29). In β-cells, MAP4K4 silencing protects against TNF-α inhibition of GSIS (11). Here we show that TM treatment increased significantly MAP4K4 gene expression in rat primary β-cells. Its silencing prevented TM action on GSIS, which seems to be TNF-α–dependent, and on β-cell proliferation and apoptosis, which is TNF-α–independent.

Inflammatory mechanisms have been suggested to contribute as causative factors in the development of type 2 diabetes. It has been further postulated that the diabetogenic environment, including insulin resistance and low-grade systemic and localized islet inflammation, contribute toward β-cell "stunning" (47). Our results lead us to propose a new component of this complex paradigm. We thus

show that induction of insulin resistance in human skeletal muscle by TNF-α leads to secretion of myokines that impact negatively on β-cell proliferation and survival. Of course, this is just one in vitro model of human skeletal muscle insulin resistance, and the panel of myokines secreted by myotubes rendered insulin resistant by other means or taken directly from insulin-resistant patients may be different from that induced by TNF-α: this is worthy of further study. At present, there is little evidence for elevated TNF-α in the skeletal muscle of individuals with type 2 diabetes, whereas its contribution toward skeletal muscle insulin resistance is well established (18). Nevertheless, assuming that it will prove possible to extrapolate from the present artificial situation with myokine concentrations in the conditioned media that are arbitrary and dependent on the in vitro conditions, to the clinical setting, the identification of these myokines and of this new pathway for interorgan communication between skeletal muscle and β-cells offers new insight into the pathological process linking insulin resistance to β-cell failure and opens the possibility for new therapeutic strategies for preservation of functional β-cell mass in type 2 diabetes. Collectively, our results regarding TM action on primary β-cells combined with earlier data in these and other cell types (11,17,29,46) place MAP4K4 as a novel target in the effort to treat or prevent diabetes by addressing both peripheral insulin resistance and the loss of β-cell mass. Our results also indicate a novel mechanism allowing for protection of β-cells by GLP-1. Finally, the beneficial effects of conditioned medium from insulin-sensitive myotubes (CMs) raises the interesting prospect of identifying novel molecules able to improve β-cell function, survival, and proliferation while suggesting that communication from skeletal muscle may contribute toward normal β-cell function and mass in healthy individuals.

ACKNOWLEDGMENTS

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