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ORIGINAL PAPER

Serum albumin protects from cytokine-induced pancreatic β cell death by a phosphoinositide 3-kinase-dependent mechanism

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Abstract The present study was undertaken to investigate the biological activity of serum albumin when pancreatic β cells were challenged by cytokines and pro-apoptotic reactive oxygen species like H_2O_2. Culture of mouse islets or INS-1E β cells for 24 h in the presence of H_2O_2 (25 μmol/l) increased cell death. This demise was prevented by serum albumin, dependent on its free sulfhydryl group, emphasizing that albumin may scavenge H_2O_2 due to its antioxidant properties. Culture for 48 h with a cytokine mixture of IL-1β (160 pg/ml), IFN-γ (200 ng/ml), and TNF-α (2 ng/ml) revealed that albumin, also protected against cytokine-induced death of both mouse islets and INS-1E β cells. This protective effect against cytokine-induced β cell death was, however, not dependent on albumins free sulfhydryl group, but was inhibited by the phosphoinositide 3-kinase (PI3K) inhibitors LY294002 (25 μmol/l) and wortmannin (1 μmol/l), suggesting that albumin may rescue β cells from cytokine-induced cell death by activation of PI3K. In accordance, albumin stimulated phosphorylation of Akt, a down-stream target for PI3K. In conclusion, it is suggested that albumin may be a survival factor for pancreatic β cells through scavenging of reactive oxygen species and by PI3K-dependent activation of Akt.

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Introduction

Insulin secreting β cells are subject to injury from oxidative stress. Formation of reactive oxygen species (ROS), such as superoxide anion (O_2^{-1}), hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\bullet}OH$), and the concomitant generation of nitric oxide (NO) have been implicated in β cell dysfunction or cell death caused by autoimmune attack and actions of cytokines in type 1 diabetes [1, 2]. ROS have also been associated with the impairment of β cell function in type 2 diabetes [2–4]. Indeed, compared with many other cell types, the β cell may be uniquely at high risk of oxidative damage and has an increased sensitivity for apoptosis [3–5].

Albumin is unique among plasma proteins in having a free sulfhydryl group at Cys^{34} [6, 7], a feature which has been shown to protect several cell types from oxidative damage through scavenging of ROS [8, 9]. With plasma levels ranging from 35 to 50 mg/ml [10], albumin is the most important antioxidant in serum. In addition, albumin is also present at appreciable, although lower levels in the extra vascular compartment [11], and cellular uptake of albumin by receptor-mediated endocytosis is well documented [12–14]. In this context, albumin binding to cell surface receptors like megalin may protect from apoptosis through activation of intracellular signaling cascades and activate PI3K and/or p44/p42 MAPK as observed in some cell types [15–19].

Albumin may thus be of significance, lowering intracellular ROS concentrations and actions in islets and probably lowering the likelihood of diabetes. The present

study was therefore undertaken to explore the capacity of bovine serum albumin in prevention of cytokine-induced and H_2O_2-induced β cell death.

Results

Serum albumin protects against H_2O_2-induced β cell death by an antioxidant mechanism

A 24 h exposure of mouse islets to H_2O_2 (25 µmol/l) caused a 23% ($P < 0.05$) reduction in islet viability, which was ameliorated in the presence of albumin (11 mg/ml) (Fig. 1).

Parallel experiments with INS-1E β cells revealed that H_2O_2 at 25 and 50 µmol/l reduced cell viability to approx. 60% ($P < 0.05$) and 10% ($P < 0.001$) of control values in a process which was antagonized by the antioxidant N-acetylcysteine (5 mmol/l) (Fig. 2a).

Albumin rescued INS-1E cells from H_2O_2-induced cell death and caused a total abolition of H_2O_2 (25 µmol/l)-induced cell death at 2.75 and 5.5 mg/ml albumin (Fig. 2a) ($P > 0.05$). After exposure to 50 µmol/l H_2O_2 cell viability was increased from approx. 8% in the absence to approx. 18% ($P < 0.01$) and 22% ($P < 0.001$) in the presence of 2.75 and 5.5 mg/ml albumin, respectively (Fig. 2b).

Free SH groups in the two batches of albumin used in the present study were determined to 0.34 and 0.36 mol per mol albumin, respectively, or approx. 30 µmol/l free SH at 5.5 mg/ml albumin. These free SH groups were totally removed by *N*-ethylmaleimide (NEM) treatment. The ability of albumin to protect against oxidative damage by H_2O_2 was reduced and did not reach statistical significance ($P > 0.05$) after treatment of albumin with NEM (Fig. 2b),

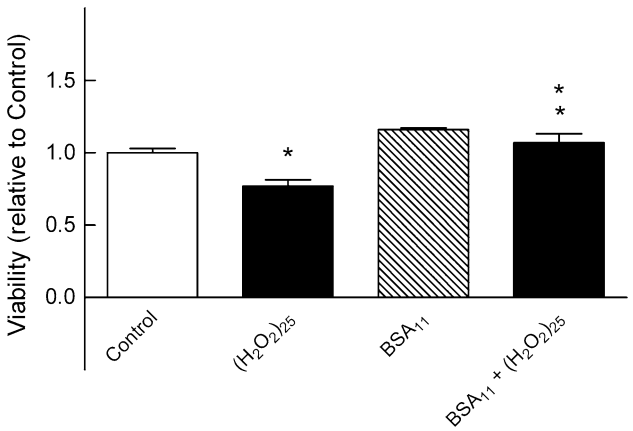

Fig. 1 Effects of H_2O_2 on cell viability of mouse islets. Islets were cultured for 24 h in the absence or presence of H_2O_2 (25 µmol/l) ($(H_2O_2)_{25}$) and with or without bovine serum albumin (11 mg/ml) (BSA_{11}) before assessment of cell viability. Results are means ± SEM ($n = 3$). *$P < 0.05$ versus Control, **$P < 0.05$ versus $(H_2O_2)_{25}$

suggesting that albumin mainly prevents H_2O_2-induced cell death due to its antioxidant properties. NEM treatment of albumin per se did not affect cell viability (Fig. 2c).

Serum albumin protects against cytokine-induced β cell death by a PI3K-dependent mechanism

Albumin also protected from cytokine-induced death of β cells (Fig. 3). Thus, a 48 h exposure to a cytokine mixture of IL-1β (160 pg/ml), IFN-γ (200 ng/ml), and TNF-α (2 ng/ml) reduced cell viability to approx. 47% ($P < 0.001$) which was ameliorated in the presence of albumin (11 mg/ml) ($P < 0.01$).

Parallel experiments with INS-1E β cells revealed that, a 48 h exposure of INS-1E cells to a cytokine mixture of IL-1β, IFN-γ, and TNF-α led to a dose-dependent inhibition of cell viability which was ameliorated in a dose-dependent fashion in the presence of serum albumin (Fig. 4a, b). Surprisingly, NEM-treated albumin retained this ability (Fig. 4c), suggesting that albumin may not rescue from cytokine-induced β cell death due to its antioxidant properties. In comparison, however, the unrelated glycoprotein ovalbumin failed to protect against cytokine-induced cell death (Fig. 4d), arguing against a non-specific protein effect in preservation β cell viability.

We therefore, aimed at investigating the possible role of PI3K and of p44/p42 MAPK in albumin signaling. A 48-h exposure to cytokines and albumin in the absence or presence of the PI3K inhibitors LY294002 (25 µmol/l) and wortmannin (1 µmol/l), which per se did not affect cell viability in the absence of albumin, revealed that inhibition of PI3K antagonized albumin protection from cytokine-induced β cell death (Fig. 5a), suggesting that albumin may activate PI3K as observed in several other cell types. Thus, in the presence of albumin (2.75 mg/ml) plus cytokines, cell viability decreased from approx. 60% in the absence to approx. 39% ($P < 0.001$) in the presence of LY294002 (25 µmol/l) and to approx. 39% ($P < 0.001$) in the presence of wortmannin (1 µmol/l). In comparison, the p44/p42 MAPK inhibitor PD98059 (25 µmol/l) failed to prevent albumin protection from cytokine-induced β cell death (Fig. 5b), suggesting that albumin may not activate this MAPK pathway in β cells. Inhibition of the pro-apoptotic p38 MAPK by SB203580 (10 µmol/l) failed to lower cytokine-induced β cell death significantly in the absence or presence of albumin (2.75 mg/ml) (Fig. 5b), suggesting that albumin may not prevent cytokine-induced cell death through a direct inhibition of p38 MAPK.

Serum albumin stimulates phosphorylation of Akt

Albumin may activate PI3K [15–18] by binding to the endocytotic receptor megalin [19], which is expressed in

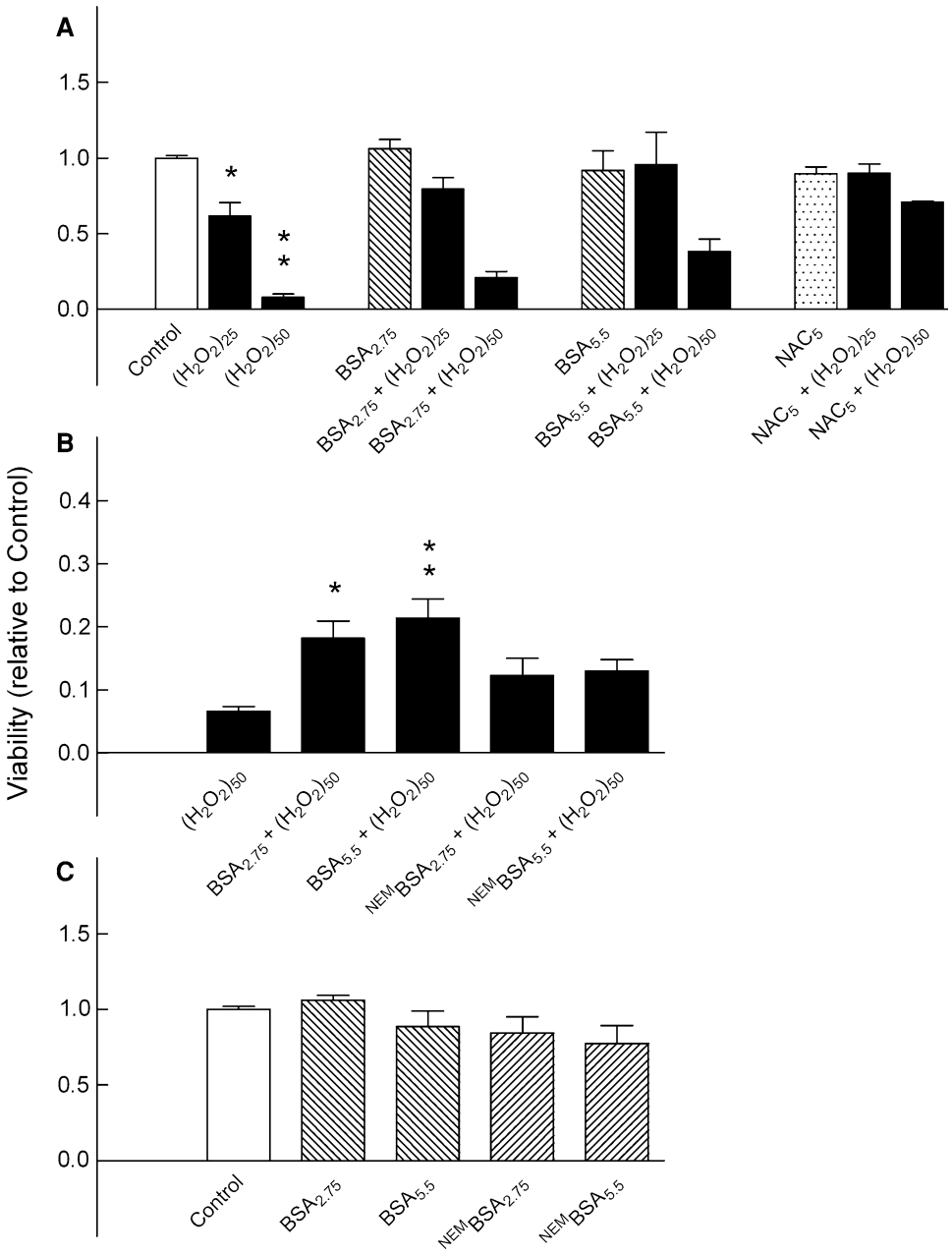

Fig. 2 Effects of H_2O_2 on cell viability of INS-1E β cells. INS-1E cells were cultured for 24 h in the absence or presence of H_2O_2 (25, 50 μmol/l) ($(H_2O_2)_{25,\ 50}$) and with or without bovine serum albumin (2.75, 5.5 mg/ml) ($BSA_{2.75,\ 5.5}$), *N*-ethylmaleimide-treated bovine serum albumin (2.75, 5.5 mg/ml) ($^{NEM}BSA_{2.75,\ 5.5}$) or *N*-acetylcysteine (5 mmol/l) (NAC_5) before assessment of cell viability. Results are means ± SEM ($n = 3$–8). (**a**) $^*P < 0.05$, $^{**}P < 0.001$ versus control, (**b**) $^*P < 0.01$, $^{**}P < 0.001$ versus $(H_2O_2)_{50}$

β cells [20]. We therefore aimed at investigating whether albumin stimulates phosphorylation of Akt a downstream target for PI3K.

A 48-h exposure of INS-1E cells to albumin (0.2 mg/ml), which exerts a strong protection against cytokine-induced β cell death (Fig. 4b), stimulated the phosphorylation of Akt at Ser-473 by approx. 76% relative to control values ($P < 0.05$) (Fig. 6a). In comparison, neither ovalbumin (0.2 mg/ml) nor exogenous insulin (25 nmol/l) appeared to stimulate phoshorylation of Akt (Fig. 6b), arguing against a nonspecific protein effect which might unmask insulin stimulation of PI3K.

In this way, albumin appeared to oppose the effect of the cytokines which lowered phosphorylation of Akt (Fig. 6b). Thus, a 48-h exposure to a cytokine mixture of IL-1β (40 pg/ml), IFN-γ (50 ng/ml), and TNF-α (0.5 ng/ml), reduced Akt phosphorylation to approx. 20% of control values ($P < 0.0001$).

Discussion

According to the present experiments the redox state of albumin may be of significance in the prevention of

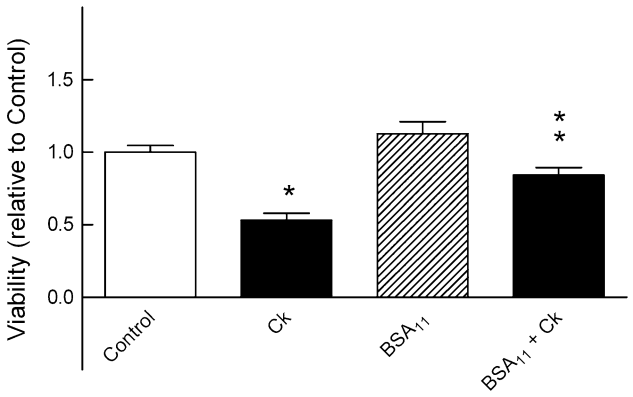

Fig. 3 Effects of cytokines on cell viability of mouse islets. Islets were cultured for 48 h in the absence or presence of cytokines (IL-1β (160 ng/ml), IFN-γ (200 ng/ml) and TNF-α (2 ng/ml)) (Ck) and with or without bovine serum albumin (11 mg/ml) (BSA_{11}) before assessment of cell viability. Results are means ± SEM ($n = 6$). *$P < 0.001$ versus control, **$P < 0.01$ versus Ck

oxidative stress-induced β cell dysfunction and death in diabetes. Albumin is unique among serum proteins in having a free sulfhydryl group at Cys^{34} and is the most important reductant in plasma and changes in the redox state may therefore be speculated to affect the risk for development of diabetes. Thus, albumin prevented both β cell death in mouse islets and clonal INS-1E β cells during exposure to H_2O_2. These protective effects of albumin appear mainly to reflect an antioxidant function of albumin, since it was obliterated by blocking of the free sulfhydryl group by *N*-ethylmaleimide.

Cytokines are known to kill β cells due to the production of ROS species including H_2O_2 and NO [1, 2]. Previous studies have suggested that antioxidants like *N*-acetylcysteine or over expression of catalase, which removes H_2O_2, may rescue some insulinoma cell lines [21, 22] although not primary β cells [23, 24] from cytokine-induced cell death. According to the present experiments, however, albumin rescue of INS-1E β cells from cytokine-induced cell death was not due to its antioxidant properties. The reason for this apparent discrepancy is not known, but it seems possible that cytokine-induced β cell death in INS-1E cells may be more dependent on NO, as suggested for primary β cells, where removal of ROS by antioxidant enzymes does not rescue from cytokine-induced cell death [24].

The anti-apoptotic effect of PI3K is well established. Thus, PI3K and its down-stream target Akt are known to phosphorylate proteins of the BcL family, preventing the release of cytochrom C and activation of caspases [25, 26]. In agreement, activation of PI3K has been described to protect β cells from both fatty acid-induced and cytokine-induced cell death [27].

Albumin has been described to activate PI3K [15–18] and p44/p42 MAPK [19] in other cells types. In addition, cytokines may activate p44/p42 MAPK [28] and p38 MAPK in β cells [28, 29]. According to the present experiments, albumin appeared to protect from cytokine-induced β cell death in INS-1E cells dependent on PI3K, whereas regulation via p44/p42 MAPK and p38 MAPK appeared to be of less importance.

Activation of PI3K has also been described to prevent H_2O_2-induced β cell death in MIN6 cells [30]. Although albumin protection against H_2O_2-induced cell death in the present study was mediated mainly by anti-oxidative

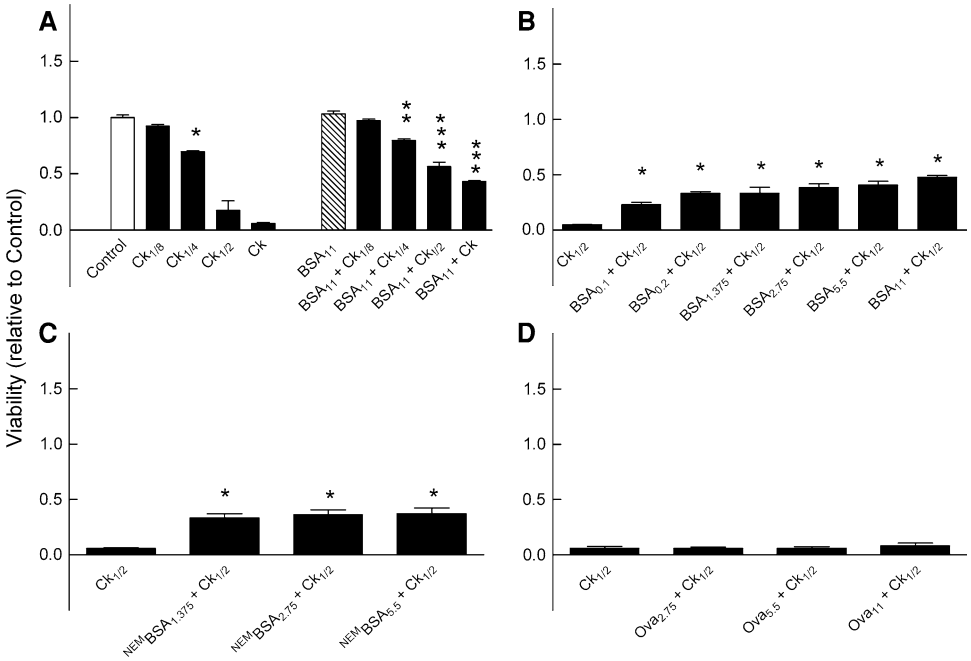

Fig. 4 Effects of cytokines on cell viability of INS-1E β cells. INS-1E cells were cultured for 48 h in the absence or presence of cytokines (IL-1β, IFN-γ, and TNF-α) (Ck), bovine serum albumin (BSA), *N*-ethylmaleimide-treated bovine serum albumin (^{NEM}BSA) or ovalbumin (Ova) as indicated. Cytokine levels in cell incubations were dilutions from a stock solution containing IL-1β (160 pg/ml), IFN-γ (200 ng/ml), and TNF-α (2 ng/ml) (Ck). Concentrations of BSA, ^{NEM}BSA, and Ova are given as mg/ml, i.e. BSA_{11} (11 mg/ml). Results are means ± SEM ($n = 3$–8). **a** *$P < 0.001$ versus control, **$P < 0.05$, ***$P < 0.001$ versus the same Ck concentration without BSA, (**b**, **c**) *$P < 0.001$ versus $Ck_{1/2}$

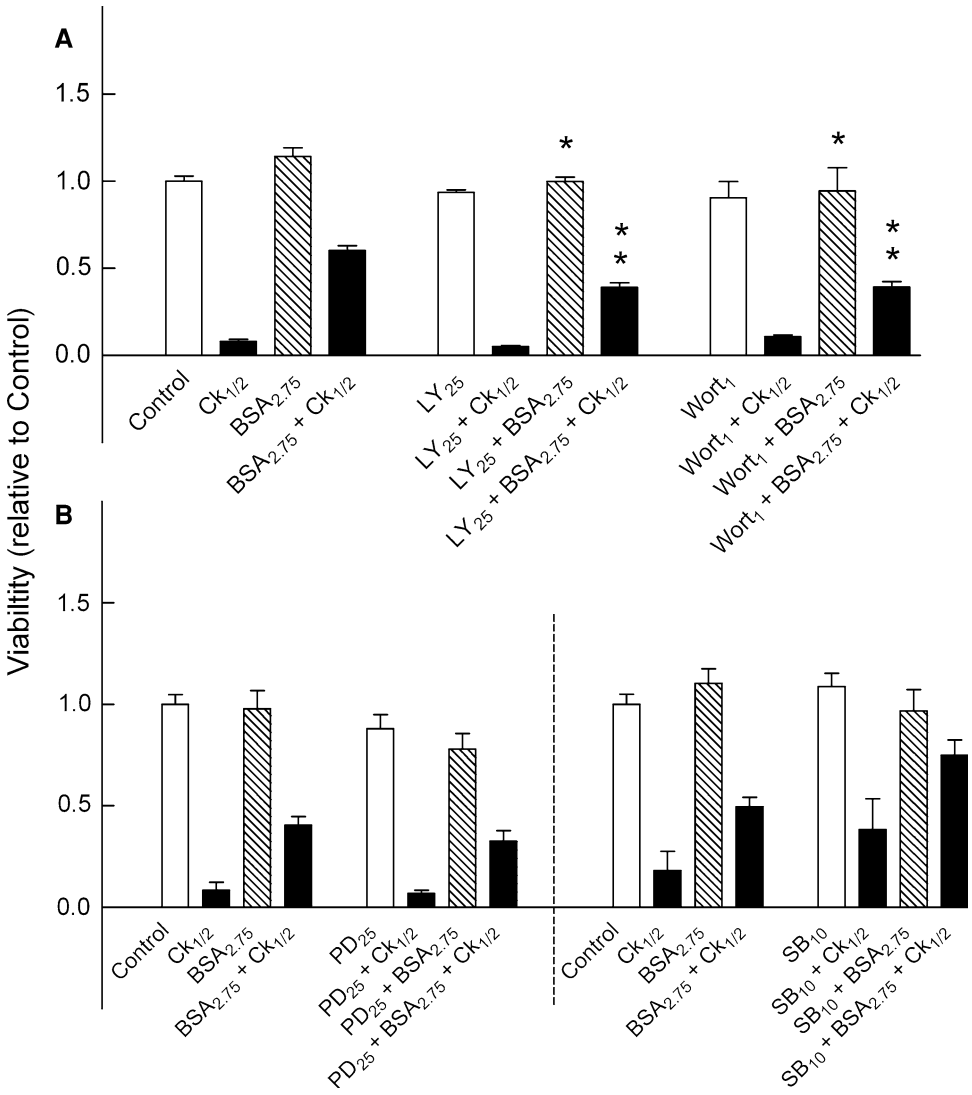

Fig. 5 Effects of PI3K and MAPK inhibitors on cytokine-induced cell death. INS-1E cells were cultured for 48 h in the absence or presence of cytokines (IL-1β (80 ng/ml), IFN-γ (100 ng/ml), and TNF-α (1 ng/ml)) ($Ck_{1/2}$), bovine serum albumin (2.75 mg/ml) ($BSA_{2.75}$) and LY294002 (25 µmol/l) (LY_{25}), wortmannin (1 µmol/l) ($Wort_1$), PD98059 (25 µmol/l) (PD_{25}) or SB203580 (10 µmol/l) (SB_{10}) as indicated. Results are means ± SEM ($n = 4$–10). *$P < 0.05$ versus $BSA_{2.75}$,**$P < 0.001$ versus $BSA_{2.75}$ + $Ck_{1/2}$

sulfhydryl groups, a contribution by PI3K can not be excluded, since NEM-albumin also appeared to retain a small albeit not significant ability to prevent H_2O_2-induced β cell death in INS-1E cells.

Insulin has previously been described to activate PI3K in β cells [31–33]. The anti-apoptotic effect of albumin does, however, not represent a secondary effect of insulin to protect against cytokine-induced β cell damage. Thus, addition of insulin failed to protect against cytokine-induced cell death (results not given) and addition of insulin did not stimulate phosphorylation of Akt, suggesting that the insulin concentration in conditioned culture media may be sufficient to account for insulin stimulation of PI3K and phosphorylation of Akt, as recently described in a study with human islets [33].

Fatty acids may propagate cytokine-induced β cell death [34]. It might therefore be speculated that albumin contrary to ovalbumin might protect against cytokines through binding of fatty acids. Preincubation of INS-1E cells for 4 h with bovine serum albumin (11 mg/ml) for extraction and binding of fatty acids [14, 35] or culture in serum free media did, however, not decrease the ensuing sensitivity to cytokines or affect the protective effect of albumin during cytokine exposure (results not given).

Most likely therefore albumin rescues from cytokine-induced β cell death through activation of PI3K. Although it may seem surprising that a protein as abundant and ubiquitous as albumin may possess signaling and anti-apoptotic functions, receptors for this protein has previously been described, including megalin, which is involved in albumin endocytosis and which stimulates the PI3K pathway [18]. Indeed β cells have previously been described to possess the components of the endocytotic pathway, and cytokines have actually been shown to increase the expression of the albumin receptor megalin [20]. Characterization of the receptor and signal transduction mechanism through which albumin mediates anti-apoptotic signaling may therefore contribute to the understanding and management of β cell viability in

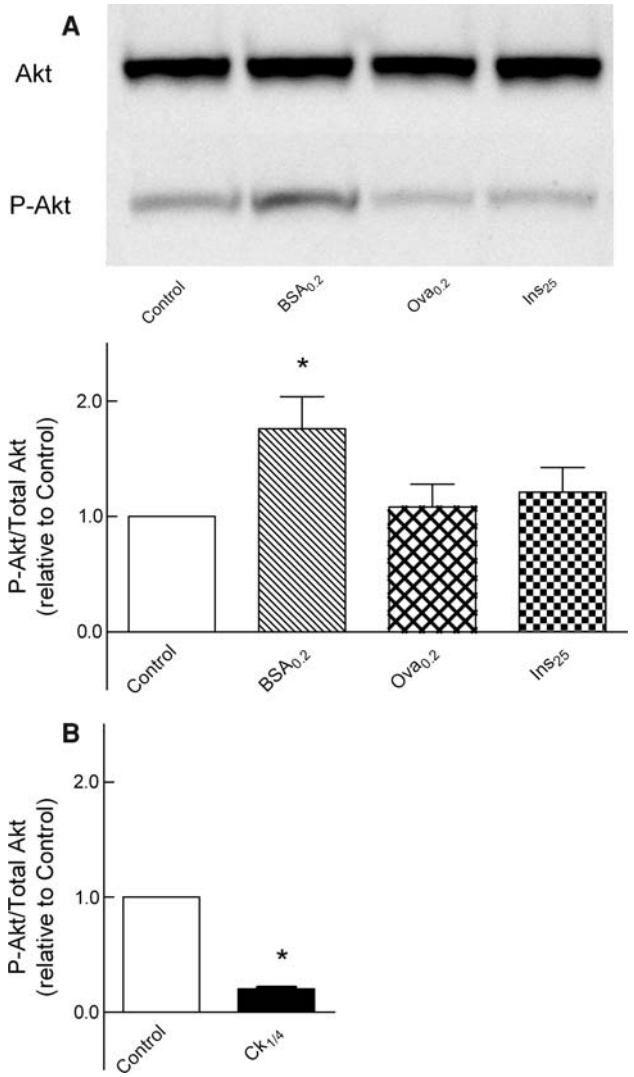

Fig. 6 Effect of albumin on Akt phosphorylation. INS-1E cells were cultured for 48 h in **a** the absence or presence of bovine serum albumin (0.2 mg/ml) ($BSA_{0.2}$), ovalbumin (0.2 mg/ml) ($Ova_{0.2}$), or insulin (25 nmol/l) (Ins_{25}) or **b** in the absence or presence of cytokines (IL-1β (40 ng/ml), IFN-γ (50 ng/ml), and TNF-α (0.5 ng/ml)) ($Ck_{1/4}$). Phosphoresidues were quantified and normalized by total Akt. Results are means ± SEM ($n = 4$). **a** $^*P < 0.05$ versus control, **b** $^*P < 0.0001$ versus control

type 1 and type 2 diabetes. This is further reinforced by the fact that albumin binding of fatty acids may obliterate albumin stimulation of PI3K and Akt [16] of potential significance for lipotoxicity and β cell failure in diabetes.

Materials and methods

Materials

Crude bacterial collagenase was obtained from Boehringer (Mannheim, Germany). Fatty acid free bovine serum albumin (BSA) (A7030) and ovalbumin (A5503) were from Sigma Chemical Co. (St. Louis, MO). LY294002, wortmannin, SB203580 and PD98059 were from Calbiochem (San Diego, CA). Recombinant mouse IL-1β was from PharMingen/BD Bioscience (San Jose, CA), recombinant rat IFN-γ and recombinant rat TNF-α were form R&D Systems (Abingdon, Oxon, UK). All other chemicals were of analytical grade. INS-1E cells were kindly provided by Claes Wollheim, Geneva.

Isolation and culture of islets

Islets were prepared by collagenase digestion of the pancreas of male albino mice (NMRI) (approx. 18–22 g body weight) (Charles River Laboratories Sulzfeld, Germany) fed ad libitum on a standard laboratory diet. Approved principles of laboratory animal care were followed. Islets were kept in tissue culture for 48–72 h in a 5% CO_2 incubator in RPMI 1640 medium (11 mmol/l glucose) supplemented with glutamax (2 mmol/l), 10% (v/v) newborn calf serum (Gibco, Paisley, Strathclyde, UK), 100 units penicillin/ml and 100 µg streptomycin/ml. Test substances were present during the last 24–48 h of culture.

Culture of INS-1E cells

INS-1E cells were cultured in 12 well plates (2.56×10^5 cells/well) or 3 cm dishes (1.25×10^6 cells/dish) in a 5% CO_2 incubator in RPMI 1640 medium (11 mmol/l glucose) supplemented with glutamax, 10% (v/v) fetal calf serum (Gibco, Paisley, Strathclyde, UK), 100 units penicillin/ml, 100 µg streptomycin/ml, and 50 µmol/l mercaptoethanol. After 48 h of culture the serum concentration was lowered to 0.5% fetal calf serum before addition of test substances for 24–48 h. This low concentration of serum did not affect cell viability.

MTT assay

The proportion of viable cells in treated versus control cells was determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, which measures succinate dehydrogenase activity [36]. Briefly mouse islets or INS-1E cells were incubated with 500 µg MTT/ml RPMI 1640 medium for 3 h at 37°C. At the end of the incubation, the medium containing MTT was removed, and the cells were dissolved in DMSO. Absorbance was then measured at 540 nm and 690 nm.

Western blot analysis

After culture INS-1E cells were washed twice in PBS++, lysed in 100 µl radioimmunoprecipitation assay (RIPA)

buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mmol/l 4-(2-aminoethyl)benzene sulfonyl fluoride, 1 mmol/l orthovanadate, 2 μmol/l okadaic acid, 1 μg/ml aprotinin, and 1 μg/ml leupeptin in PBS) and centrifuged at 11,000×*g* for 3 min. Protein concentration was then determined in supernatants using the BCA protein assay (Sigma Chemical Co., St. Louis, MO). The proteins were separated by electrophoresis on 7% NuPage Novex Tris-Acetate gels in Tris-Acetate running buffer (Invitrogen, Taastrup, Denmark) and transferred to nitrocellulose membranes by electroblotting. After incubation of the membrane in blocking buffer (Tris-buffered saline containing 5% nonfat dry milk), the membrane was exposed overnight at 4°C to rabbit Akt and Phosho-Akt (Ser-473) antibody (Cell Signaling Technology, Danvers, MA) diluted 1:1000 in blocking buffer. The membrane was subsequently washed in Tris-buffered saline containing 0.1% Tween 20, incubated with peroxidase-conjugated goat antirabbit IgG (Dako, Glostrup, Denmark), and proteins detected by chemiluminescence using ECL plus Western blotting detection reagent (GE Healthcare, Chalfont St. Giles, UK).

Determination of free thiol groups

Free thiol groups were measured using a modification of Ellman's method [37]. BSA (36 mg/ml, 10 μl) was incubated with 5,5′-dithiobis-(2-nitrobenzoic acid) (1 mg/ml, 100 μl) for 20 min and the absorbance at 410 nm was measured. *N*-ethylmaleimide (NEM) (40 mmol/l, 10 μl) was then added and the absorbance read again after 10 min. The calculation of free thiol groups was carried out as described [37] using a molar absorption coefficient of 14173 M^{-1} cm^{-1} [38]. The protein concentration of pure albumin samples was determined from the absorbance at 280 nm.

Modification of the thiol group

An aliquot of 1 ml bovine serum albumin (200 mg/ml) was incubated with dithiothreitol (5 mmol/l) for 1 h at 37°C in 25 mmol/l HEPES buffer, pH 7.4. Then 1 ml NEM (40 mmol/l) was added and the mixture was incubated for a further 1 h. Before measurement of activity, excess reagent was removed by extensive dialysis and the change in thiol group was measured as above.

Miscellaneous

LY294002, wortmannin, SB203580, and PD98059 were added in a small volume of DMSO, final conc. 0.01–0.1%. Results are given as means ± SEM for *n* experiments. Statistical evaluation of the data was made by ANOVA, followed by the Newman-Keuls test for multiple comparisons; not significant, $P > 0.05$.

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