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**Steroid effects on intracellular degradation of insulin and crinophagy in
isolated pancreatic islets**

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Abstract

Under physiological conditions substantial amounts of hormone may be degraded within endocrine cells by a crinophagic process comprising fusions of secretory granules with lysosomes. Glucocorticoids may stabilise and progesterone destabilise lysosomal membranes. The effects of corticosterone and progesterone on intracellular degradation of insulin and crinophagy were determined in pancreatic β -cells, and possible pathways mediating these effects were evaluated. Pancreatic islets were isolated from mice, intracellular degradation of insulin was measured by a pulse-chase method, and crinophagy was studied by electron microscopy. The islets were exposed to 3.3, 5.5 or 28 mM glucose with or without corticosterone, progesterone or the receptor ligands A-224817.0 and WAY-161358. Mifepristone was used to block steroid receptors and indomethacin to inhibit prostaglandin synthesis. Corticosterone caused a concentration-dependent decrease of insulin degradation at the lower glucose concentrations. Progesterone effected a concentration-dependent stimulation of insulin degradation. These results were paralleled with changes of the crinophagic activity in the β -cells. Corticosterone decreased and progesterone increased islet production of prostaglandin E₂. Mifepristone abolished the steroid actions on insulin degradation and prostaglandin production. The effects of corticosterone were mimicked by the selective glucocorticoid receptor modulator A-224817.0, but in contrast to progesterone, the selective progesterone receptor agonist WAY-161358 had no effect on insulin degradation or prostaglandin production. Inhibition of cyclooxygenase blocked insulin degradation. The findings indicate that both corticosterone and progesterone could affect intracellular insulin degradation and crinophagy solely via the glucocorticoid receptor, and that prostaglandins may have a regulatory role in intracellular turnover of secretory material in pancreatic islet β -cells.

Keywords: Pancreatic islets; Corticosterone; Progesterone; Lysosomes; Intracellular insulin degradation; Prostaglandin E₂

1. Introduction

Endocrine cells that produce polypeptide hormones maintain a rich supply of secretory granules, which enables an instant response to a stimulus. Hormone that is not released may be subjected to intracellular degradation. Indeed, ultrastructural observations on prolactin-producing cells (Smith and Farquhar, 1966) have indicated that secretory granules can be incorporated into lysosomes and degraded by a process denominated crinophagy (de Duve, 1969). At varying functional demands a relative overproduction of secretory material could be controlled by crinophagy. This notion is supported by observations of glucose-dependent alterations of the lysosomes in the insulin producing pancreatic β -cells (Schnell and Borg, 1985; Schnell et al., 1988; Schnell Landström et al., 1991).

However, little is known about the mechanism of the crinophagic process and its correlation to intracellular degradation of polypeptide hormones. Early experiments with isolated lysosomes have shown that glucocorticoids may stabilise lysosomal membranes (Weissmann, 1965; Miller and Smith, Jr., 1966; Symons et al., 1969), whereas other steroids, such as progesterone, may make lysosomal membranes unstable (Weissmann, 1965). Provided that crinophagy results in degradation of secretory material, a glucocorticoid and progesterone would consequently have opposite actions on both crinophagy and intracellular hormone degradation.

This hypothesis was tested by studies of the effects of corticosterone and progesterone on the β -cell lysosomes and on the intracellular degradation of insulin in isolated pancreatic islets. Alternative pathways for the action of the steroids were evaluated by experiments utilising mifepristone, which is an antagonist for both the glucocorticoid and the progesterone receptor (Baulieu, 1991; Agarwal, 1996; Cadepond et al., 1997), A-224817.0 that is a selective nonsteroidal glucocorticoid receptor modulator maintaining high transrepressive activity whilst having low transactivation effect (Elmore et al., 2001) and WAY-161358, which is a nonsteroidal progesterone receptor agonist virtually lacking any cross-reactivity with the glucocorticoid receptor (Zhang et al., 2003). Furthermore, the effects of the steroids on prostaglandin production in the islets were determined, and a possible role for prostaglandins as mediators of intracellular hormone degradation was assessed by experiments with indomethacin, which is an inhibitor of prostaglandin synthesis (Ferreira et al., 1971; Vane, 1971).

2. Materials and methods

2.1. Islet isolation and intracellular degradation of insulin

Pancreatic islets were isolated from adult male NMRI mice (Scanbur BK, Sollentuna, Sweden) by a collagenase digestion procedure (Schnell et al., 1988).

Intracellular degradation of insulin was determined by a pulse-chase method. The islets were incubated under tissue culture conditions in TCM 199 with Hanks' salts supplemented with 100 ml/l heat-inactivated (56 °C, 30 min) fetal bovine serum (Sigma-Aldrich Corp., St. Louis, MO, USA), 170 µM sodium benzylpenicillinate and 170 µM streptomycin. To label islet proteins during a 72-h pulse period, groups of about 150 islets were maintained in medium containing 5.5 mM glucose and 1.5 GBq/l L-[4,5-³H]leucine (GE Healthcare Life Sciences, Chalfont St. Giles, Buckinghamshire, UK) giving a specific radioactivity of 3.3 GBq/mmol. The radioactive labelling of immunoprecipitable insulin in the islets increased during the first two days of incubation and then reached a steady state.

After the incorporation of radioactive leucine, the islets were rinsed two times in non-radioactive medium and two batches of 15 islets were homogenised by sonication (22 kHz, 50 W) for 10 s in 200 µl of a 50 mM glycine buffer, pH 8.8, containing 2.5 g/l bovine serum albumin (BSA), and frozen. The remaining islets were divided into three aliquots and subjected to a chase-incubation for 24 h without radioactivity at 3.3, 5.5 or 28 mM glucose. Corticosterone, progesterone, mifepristone and indomethacin, which all were obtained from Sigma-Aldrich Corp., St. Louis, MO, USA, A-224817.0 (2,5-dihydro-10-methoxy-2,2,4-trimethyl-5-(2-propenyl)-1H-[1]benzopyrano[3,4-f]quinoline) from Abbott Laboratories, Abbott Park, IL, USA, and WAY-161358 (4-(1,4-dihydro-4,4-dimethyl-2-thioxo-2H-3,1-benzoxazin-6-yl)-2-thiophenecarbonitrile) from Wyeth Pharmaceuticals, Collegeville, PA, USA, were supplied to the incubations by addition of 5 or 10 µl from stock solutions of the substances dissolved in ethanol. It was established that the addition of 10 µl ethanol to the islet incubations neither affected immunoreactive insulin in islet homogenates nor in incubation media. After the chase period, duplicate groups of 15 islets from each glucose concentration were homogenised by sonication in 200 µl glycine-BSA-buffer, and 100-µl portions of the incubation media were also retrieved. The islet homogenates and incubation media were kept frozen until immunoprecipitation.

Portions of 10 µl from the islet homogenates and the 100-µl medium samples were mixed with 100 µl of a 50 mM glycine buffer, pH 8.8, supplemented with 2.5 g/l BSA and 1 ml/l Triton[®] X-100, and in duplicates supplied with 10 µl of guinea pig anti-human insulin

serum (Chemicon International, Temecula, CA, USA) or 10 μ l normal guinea pig serum (Harlan Sera-Lab, Loughborough, Leicestershire, UK). The immune binding was allowed to proceed for 1 h at room temperature. Subsequently 50 μ l rProtein-A-Sepharose™ Fast Flow suspended in glycine-BSA-Triton® X-100 buffer to a concentration of 110 ml/l was added, and the material was tumbled for 15 min at room temperature and then centrifuged for 3 min at 4000 x g. The Sepharose™ sediment with the anti-insulin-insulin complex was washed twice with 500 μ l glycine-BSA-Triton® X-100 buffer and finally resuspended in 500 μ l 1 M acetic acid with 2.5 g/l BSA. After addition of 2.5 ml Ultima Gold™ scintillation fluid, the radioactivity was determined by a liquid-scintillation spectrometer (Wallac System1400™) using external standardisation. Nonspecific binding was corrected for by subtracting the radioactivity bound by normal guinea pig serum from that precipitated by immune serum.

To check if any extracellular degradation of insulin occurred in the media during the chase period two control experiments were performed. Firstly, incubation media were supplied with 5 - 10 ng/ml of crystalline mouse insulin (Novo Research Institute, Bagsvaerd, Denmark) and the recovery was measured by radioimmunoassay (Heding, 1972). Secondly, immunoprecipitable porcine [¹²⁵I]iodoinsulin (PerkinElmer Life and Analytical Sciences, Wellesley, MA, USA) was determined in cultures containing 40 islets/dish as described above. No significant extracellular insulin degradation was detected by any method for up to three days irrespective of the glucose concentration or additions of corticosterone or progesterone.

2.2. Electron microscopy and enzyme cytochemistry

Islets from the chase incubations were prepared for electron microscopy and visualisation of acid phosphatase activity as described in detail elsewhere (Schnell and Borg, 1985, and ultrastructural studies were performed by a H-7100 transmission electron microscope from Hitachi, Tokyo, Japan.

2.3. Measurement of prostaglandin E₂

The content of prostaglandin E₂ (PGE₂) in the islet incubation media was measured by a competitive ELISA (GE Healthcare Life Sciences, Uppsala, Sweden).

2.5. Statistical analysis

All statistics was based on independent observations. Intracellular degradation of insulin at the various experimental conditions was evaluated by *t*-statistics. Differences between the experimental groups were assessed by factorial analysis of variance in combination with Fisher's protected least significant difference test.

3. Results

3.1. Glucose affects intracellular degradation of insulin

Intracellular degradation of insulin in the pancreatic islets was determined by a pulse-chase method. After the chase period, the content of radioactively labelled insulin in the islets and incubation media was measured and calculated as a percentage of the radioactive insulin in the islets retrieved immediately after the pulse incubation. Lacking insulin was regarded as degraded intracellularly, since no insulin degradation occurred in the incubation media under the present conditions.

The glucose concentration of the incubation medium influenced the fate of the labelled immunoprecipitable insulin during the chase period (Table 1). At 3.3 and 5.5 mM glucose the insulin secretion was low, and a lot of insulin remained in the islet β -cells. Under these circumstances more than a third of the insulin was subjected to intracellular degradation ($p < 0.001$). When the islets were exposed to 28 mM glucose, the insulin secretion was greatly enhanced and the islet insulin content decreased. However, the total insulin content of the chase incubations was markedly increased. Hence, at a glucose concentration of 28 mM there was no intracellular degradation of insulin ($p > 0.05$).

3.2. Corticosterone decreases and progesterone increases intracellular degradation of insulin

The addition of corticosterone or progesterone to the chase medium changed the islet handling of the labelled insulin (Fig. 1). Corticosterone caused a concentration-dependent decrease of the islet intracellular degradation of insulin. Quite the opposite was observed, when the medium was supplemented with progesterone. This resulted in a concentration-dependent stimulation of the intracellular degradation of insulin. Even at 28 mM glucose, the additions of progesterone resulted in an intracellular degradation of about a third to one-half of the labelled insulin.

3.3 The effects of glucose and steroids on intracellular degradation of insulin are paralleled with structural changes of the β -cell lysosomes

When the pancreatic islets were incubated with 3.3 or 5.5 mM glucose, their β -cells showed a conspicuous supply of secretory granules, whereas they displayed a low content of granules at 28 mM glucose. There was a great variation in the ultrastructure of the lysosomes of the islet β -cells. At a glucose concentration of 3.3 and 5.5 mM most lysosomes had a heterogeneous content with various kinds of inclusions (Fig. 2A). Many of these lysosomes appeared as multigranular bodies enclosing material resembling secretory granules (Fig. 2B). These lysosomes indicated an active crinophagic process. The lysosomes predominating in the β -cells of the islets incubated with 28 mM glucose exhibited a homogenous, very fine, particulate content of high density and a narrow light rim, or halo, just beneath their surrounding membrane (Fig. 2C). These lysosomes were defined as dense bodies and showed no signs of involvement in crinophagy.

The addition of corticosterone to the incubation medium resulted in an increased content of secretory granules in the islet β -cells. Most β -cell lysosomes in the islets treated with corticosterone appeared as dense bodies also at the lower glucose concentrations (Fig.3A). The dense bodies were entirely filled with cytochemically reactive acid phosphatase (Fig.3A, inset). Progesterone caused an apparent decrease in the number of secretory granules in the β -cells of the pancreatic islets. Numerous large multigranular bodies could be seen in the β -cells exposed to progesterone at all three glucose concentrations. Notably, there was a considerable number of β -cell multigranular bodies in the islets treated with progesterone at 28 mM glucose (Fig. 3B). These lysosomes demonstrated always a patchy distribution of acid phosphatase activity (Fig. 3B, inset). The observations implied that corticosterone and progesterone exerted divergent effects on the lysosomal population of the islet β -cells. Corticosterone obviously decreased, whereas progesterone increased, the crinophagic activity of these cells.

3.4. Mifepristone abolishes the effects of steroids on intracellular degradation of insulin

The effects of corticosterone and progesterone on the intracellular degradation of insulin were moreover evaluated by experiments with mifepristone (Fig. 4). In incubations with 3.3 or 5.5 mM glucose, 20 μ M mifepristone had in itself no effect on islet intracellular insulin degradation. However, when mifepristone was added to incubations containing 20 μ M

corticosterone or 20 μ M progesterone at these glucose concentrations, the insulin degradation was restored to the same level as in the control islets. The effects of mifepristone were less clear at 28 mM glucose.

3.5. A-224817.0 decreases whereas WAY-161358 has no effect on intracellular degradation of insulin

Involvement of steroid receptors in the effects of corticosterone and progesterone on the intracellular insulin degradation was further analysed by use of selective nonsteroidal receptor ligands (Fig. 5). At 3.3 or 5.5 mM glucose, A-224817.0 at a concentration of 5 μ M, in similarity to corticosterone, reduced the intracellular insulin degradation. On the contrary, 10 μ M WAY-161358 did not affect the degradation at these concentrations of glucose. In incubations with 28 mM glucose, none of the ligands caused any change in the degradation of insulin.

3.6. Specific receptor effects decrease and increase islet production of prostaglandin E₂

The islet production of prostaglandins was determined by measurements of PGE₂ accumulation in the chase medium at a glucose concentration of 5.5 mM. In the presence of 20 μ M corticosterone the islet PGE₂ production was decreased and with 20 μ M progesterone increased. Mifepristone at a concentration of 20 μ M did not change the production of PGE₂. However, this substance abolished the effects of corticosterone and progesterone on the islet PGE₂ production (Fig. 6).

The effect of corticosterone was mimicked with 5 μ M A-224817.0 that also reduced the PGE₂ production. On the other hand, 10 μ M WAY-161358 had no effect on the islet production of the prostaglandin (Fig. 7).

3.7. Inhibition of cyclooxygenase blocks intracellular degradation of insulin

To estimate the role of prostaglandins for the intracellular degradation of insulin, the effects of indomethacin were determined (Fig. 8). The intracellular degradation of insulin that occurred in the islets at 3.3 and 5.5 mM glucose was abolished, when 100 μ M indomethacin was added to the chase medium. At 28 mM glucose, where there was no intracellular insulin degradation, indomethacin did not have any further effect.

Discussion

This study showed a substantial intracellular degradation of radioactively labelled insulin in the pancreatic islets at low or physiologic glucose concentrations, whereas there was no degradation at a high glucose concentration. These results are in agreement with previous observations (Halban and Wollheim, 1980). Corticosterone inhibited and progesterone stimulated the intracellular degradation of insulin in the islets. The effects were detected at a steroid concentration of 1 μM , which is five to ten times higher than the normal plasma concentration in mice (Halberg et al., 1959; Solem, 1966; Michael, 1976; Bailey, 1987). However, in the present experiments the isolated islets had lost their microcirculation, and it is plausible that they had a low tissue extraction of the steroids.

Corticosterone and progesterone had divergent actions on the lysosomal population of the pancreatic β -cells. The lysosomes, which were identified by their content of acid phosphatase, appeared in the β -cells exposed to corticosterone mostly as dense bodies not involved in any degradative process. However, in the islets treated with progesterone the lysosomal population of the β -cells was predominated by large multigranular bodies. Hence, both the effects of the various glucose concentrations and of corticosterone and progesterone indicate an apparent concordance between intracellular degradation of insulin and crinophagy in the pancreatic β -cells.

Conceivably, the actions of the steroids on the intracellular degradation of insulin and crinophagy in the islet β -cells could be attributed to physicochemical effects on the lysosomal and secretory granule membranes. Some studies on artificial membrane models have shown opposite actions of this kind by glucocorticoids and progesterone (Roux and Baret, 1968; Heap et al., 1970), whereas other studies have not revealed any differing effects of glucocorticoids and progesterone (Kellaway and Saunders, 1967) and in particular not of corticosterone and progesterone (Bangham et al., 1965). As an alternative, it may be considered that the steroid effects could be mediated by specific membrane receptors (Wehling, 1997; Lösel and Wehling, 2003; Norman et al., 2004). However, mifepristone, which is an antagonist to the classical cytoplasmic receptors for glucocorticoids and for progesterone (Baulieu, 1991; Agarwal, 1996; Cadepond et al., 1997), abolished the effect of the steroids on the intracellular degradation of insulin. Thus, it is suggested that the action of these receptors mediate the effects of corticosterone and progesterone on the intracellular insulin degradation and crinophagy in the pancreatic islets. Both the glucocorticoid receptor

(Fischer et al., 1990; Matthes et al., 1994) and the progesterone receptor (Green et al., 1978; Doglioni et al., 1990) are present in the β -cells. However, it is known that progesterone may bind to the glucocorticoid receptor with an affinity similar to the natural glucocorticoids (Chader and Reif-Lehrer, 1972; Moguilewsky and Deraedt, 1981; Rupprecht et al., 1993). When the pancreatic islets were exposed to the selective nonsteroidal receptor ligands at concentrations equipotent to those of the steroids, WAY-161358, which is a strong progesterone receptor agonist virtually without any cross-reactivity with the glucocorticoid receptor (Zhang et al., 2003), had in contrast to progesterone no effect on the intracellular degradation of insulin. On the other hand, the effect of corticosterone was mimicked by A-224817.0, which has a high affinity for the glucocorticoid receptor (Elmore et al., 2001). Taken together these findings may indicate that both corticosterone and progesterone could affect intracellular degradation of insulin solely via the glucocorticoid receptor.

In all the present experiments there was a close correlation between islet intracellular insulin degradation and islet production of PGE₂. Both corticosterone and A-224817.0, with its high transrepressive activity (Elmore et al., 2001), inhibited the islet PGE₂ production. In combination with the *in silico* prediction of the cyclooxygenase-2 gene as a target for the transcription factor NF- κ B in the pancreatic β -cells (Naamane et al., 2007), these observations suggest that glucocorticoids alter islet β -cell prostaglandin production via receptor-regulated NF- κ B activity, as earlier described in other cell types (Barnes, 1998; McKay and Cidlowski, 1999; De Bosscher et al., 2003). Since progesterone, but not the progesterone receptor agonist WAY-161358, enhanced the islet production of PGE₂, it is tempting to speculate that progesterone in the pancreatic β -cells facilitates NF- κ B activity via binding to the glucocorticoid receptor. It has been observed that progesterone bound to the glucocorticoid receptor may exert both transactivation and transrepression (Koubovec et al., 2005), and maybe the steroid changes the ratio between the two processes in the pancreatic β -cells.

In summary, corticosterone and progesterone may exert their effects on islet intracellular insulin degradation and crinophagy by their control of prostaglandin production. The finding that indomethacin, an inhibitor of prostaglandin synthesis (Ferreira et al., 1971; Vane, 1971), abolished the islet intracellular degradation of insulin supports this notion and, furthermore, suggests a regulatory role for prostaglandins in intracellular turnover of secretory material in pancreatic islet β -cells.

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Figure legends

Fig. 1. Effects of corticosterone or progesterone on intracellular degradation of insulin in pancreatic islets at 3.3 (A), 5.5 (B) or 28 (C) mM glucose. Results are given as mean values \pm S.E.M. for 8 - 10 observations. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ indicate probability of chance for no insulin degradation at each experimental condition. $\dagger p < 0.05$, $\dagger\dagger p < 0.01$, $\dagger\dagger\dagger p < 0.001$ versus control islets.

Fig. 2. Effects of glucose on pancreatic islet ultrastructure. (A) A β -cell at 3.3 mM glucose showing a lysosome with heterogeneous inclusions partly resembling membrane fragments. (B) A β -cell at 5.5 mM glucose with a multigranular body containing secretory granules. (C) A β -cell at 28 mM glucose displaying a dense body with homogenous, very fine, particulate content and a narrow light rim just beneath the surrounding membrane. Magnification 33 900 \times .

Fig. 3. Effects of corticosterone or progesterone on pancreatic islet ultrastructure. (A) A β -cell exposed to 100 μ M corticosterone at 3.3 mM glucose showing a dense body. (B) A β -cell exposed to 100 μ M progesterone at 28 mM glucose with a multigranular body. Magnification 33 900 \times . Insets show cytochemically reactive acid phosphatase completely filling a dense body (A) or displaying a patchy distribution within a multigranular body (B). Magnification 51 000 \times .

Fig. 4. Effects of corticosterone or progesterone and/or mifepristone on intracellular degradation of insulin in pancreatic islets at 3.3 (A), 5.5 (B) or 28 (C) mM glucose. Results are given as mean values \pm S.E.M. for 8 - 10 observations. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ indicate probability of chance for no insulin degradation at each experimental condition. $\dagger p < 0.05$, $\dagger\dagger p < 0.01$, $\dagger\dagger\dagger p < 0.001$ versus control islets.

Fig. 5. Effects of A-224817.0 or WAY-161358 on intracellular degradation of insulin in pancreatic islets at 3.3 (A), 5.5 (B) or 28 (C) mM glucose. Results are given as mean values \pm S.E.M. for 8 - 10 observations. * $p < 0.05$, *** $p < 0.001$ indicate probability of chance for no insulin degradation at each experimental condition. $\dagger\dagger\dagger p < 0.001$ versus control islets.

Fig. 6. Effects of corticosterone or progesterone and/or mifepristone on prostaglandin E₂ production of pancreatic islets at 5.5 mM glucose. Results are given as mean values ± S.E.M. for 12 - 15 observations. ††† $p < 0.001$ versus control islets.

Fig. 7. Effects of A-224817.0 or WAY-161358 on prostaglandin E₂ production of pancreatic islets at 5.5 mM glucose. Results are given as mean values ± S.E.M. for 7 - 9 observations. †† $p < 0.01$ versus control islets.

Fig. 8. Effects of indomethacin on intracellular degradation of insulin in pancreatic islets at 3.3, 5.5 or 28 mM glucose. Results are given as mean values ± S.E.M. for 6 observations. *** $p < 0.001$ indicate probability of chance for no insulin degradation at each experimental condition. †† $p < 0.01$, ††† $p < 0.001$ versus control islets at each glucose concentration.

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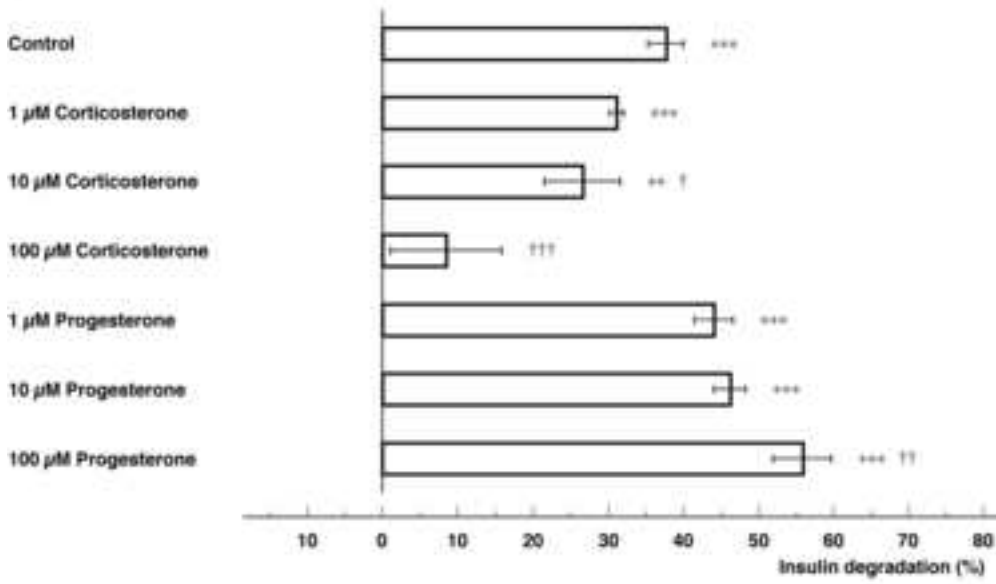
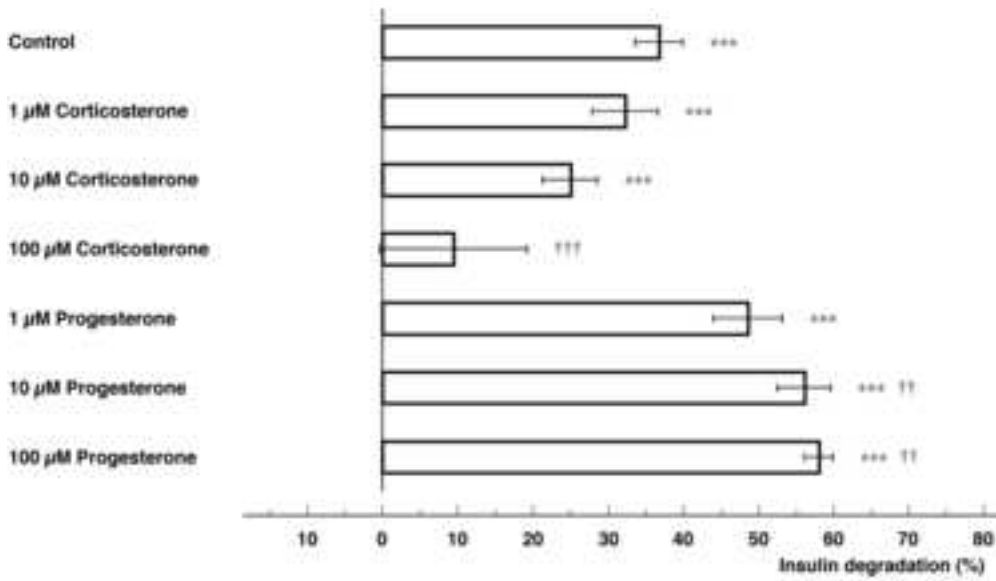
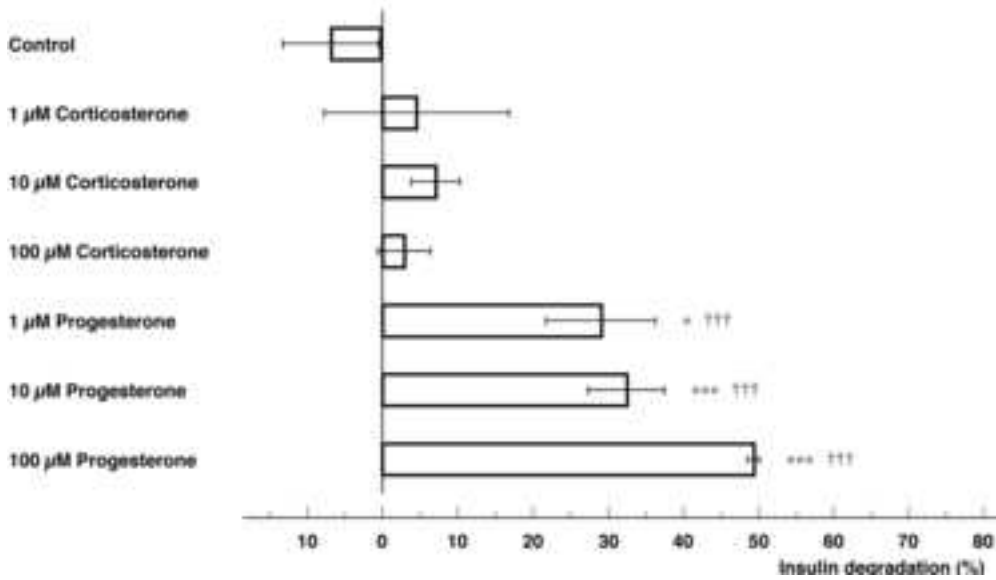
Table 1 Effects of glucose on insulin in pancreatic islets

Glucose concentration (mM)	Islet insulin content (%)	Medium insulin content (%)	Total insulin (%)	Degraded insulin (%)
3.3	60.6 ± 2.4	2.4 ± 0.8	62.3 ± 2.4	37.7 ± 2.4
5.5	59.8 ± 3.3	3.8 ± 0.7	63.1 ± 3.2	36.9 ± 3.2
28	24.8 ± 1.1***	79.3 ± 6.3***	106.6 ± 6.4***	-6.6 ± 6.4

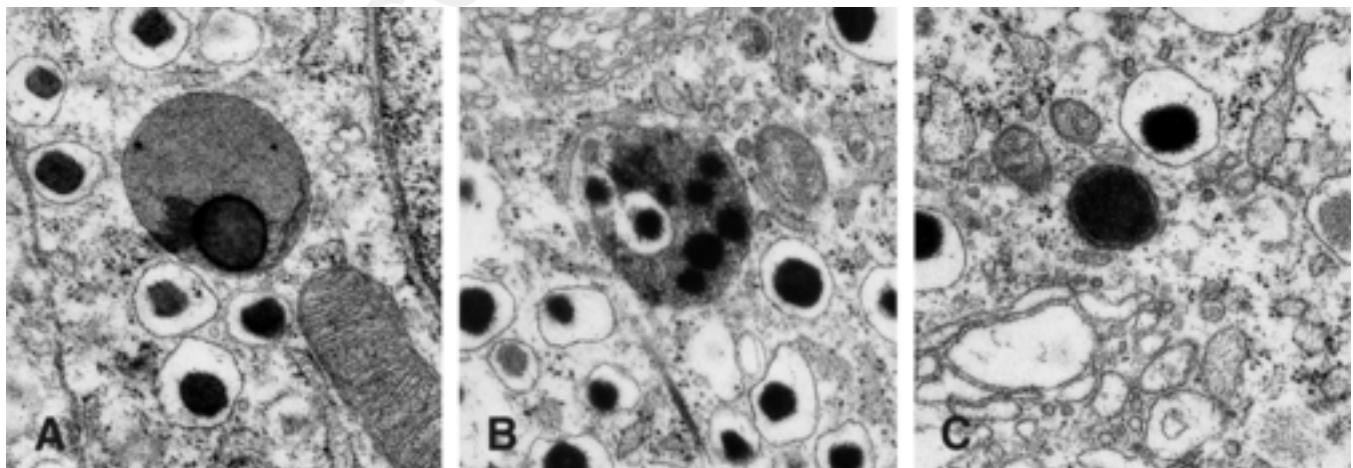
Results are given as mean values ± S.E.M. for 10 observations.

*** $P < 0.001$ versus islets incubated at 5.5 mM glucose.

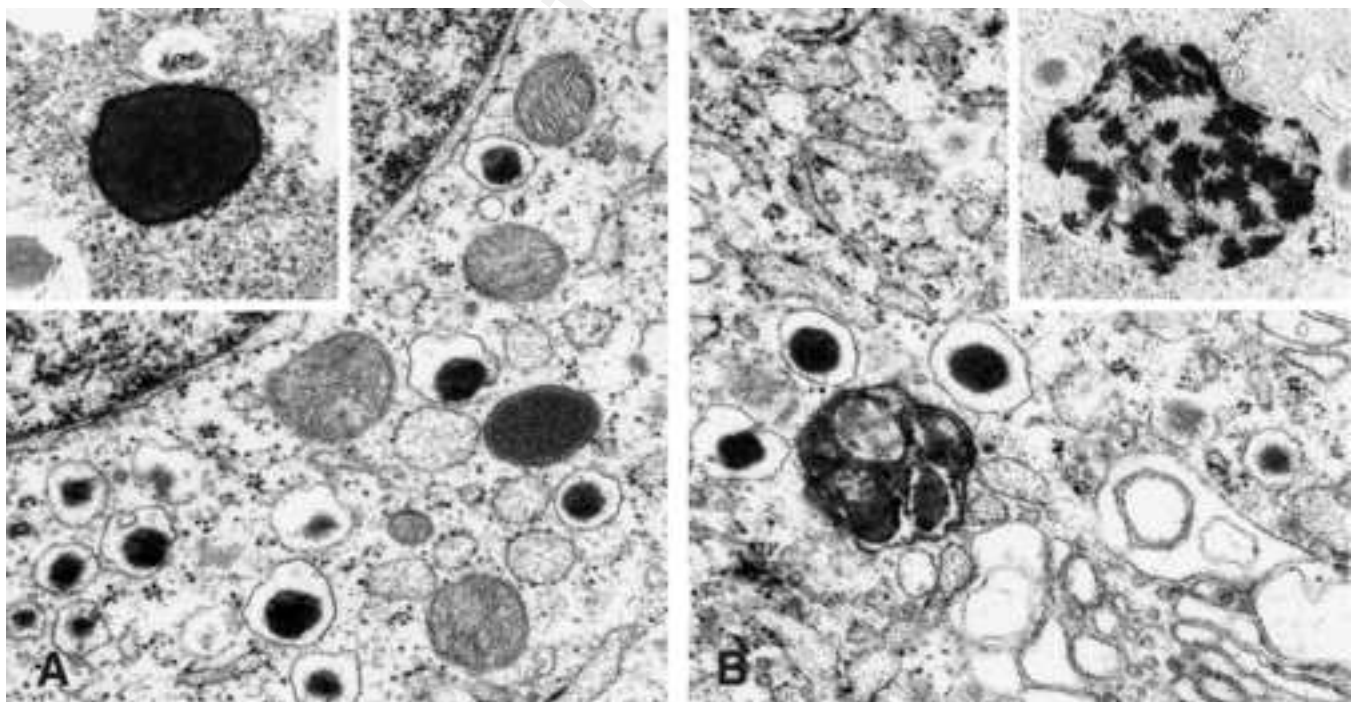
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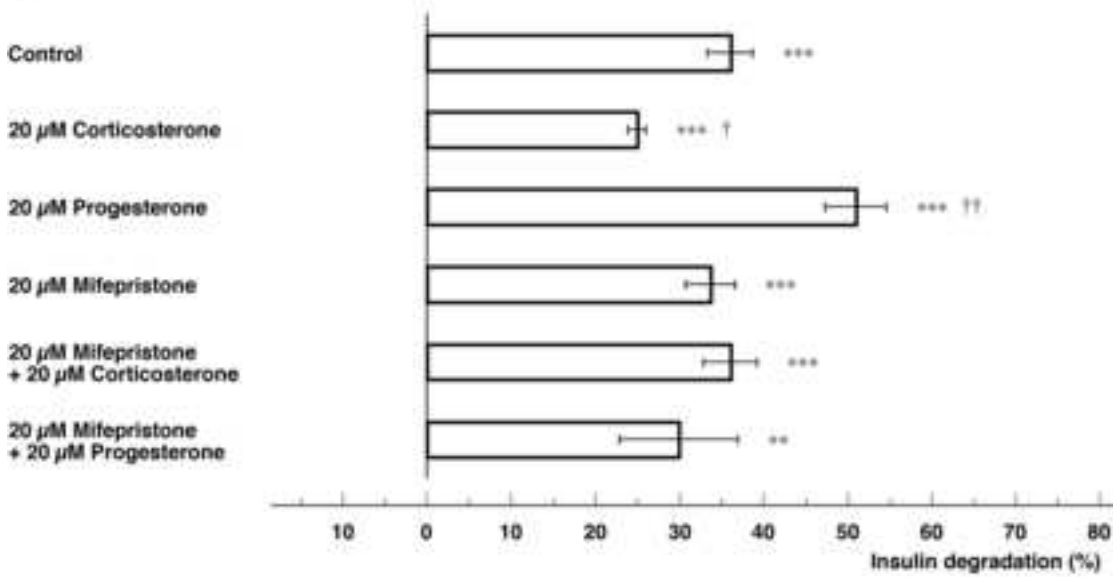
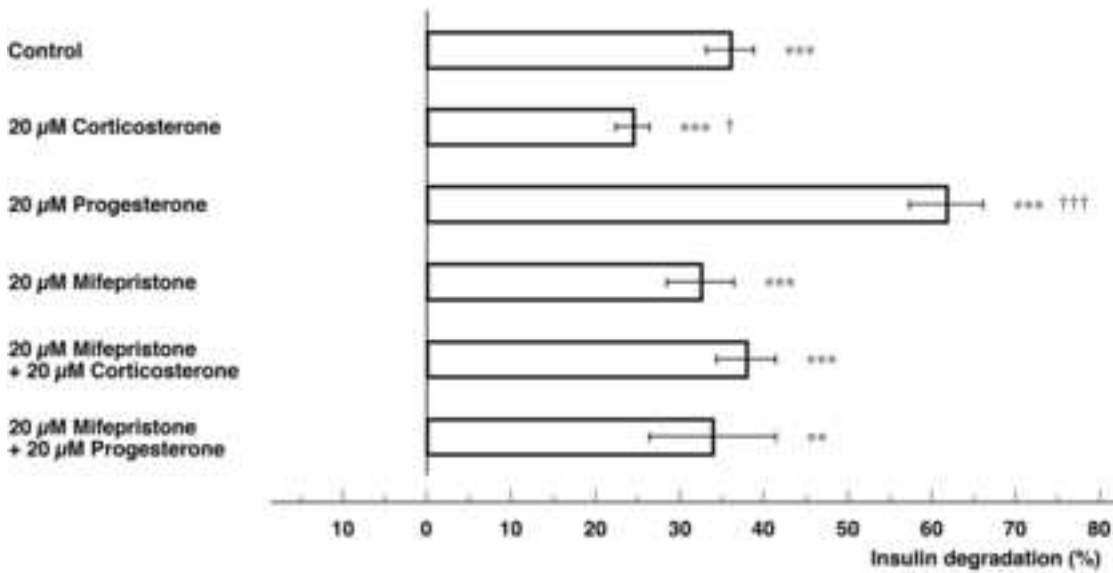
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